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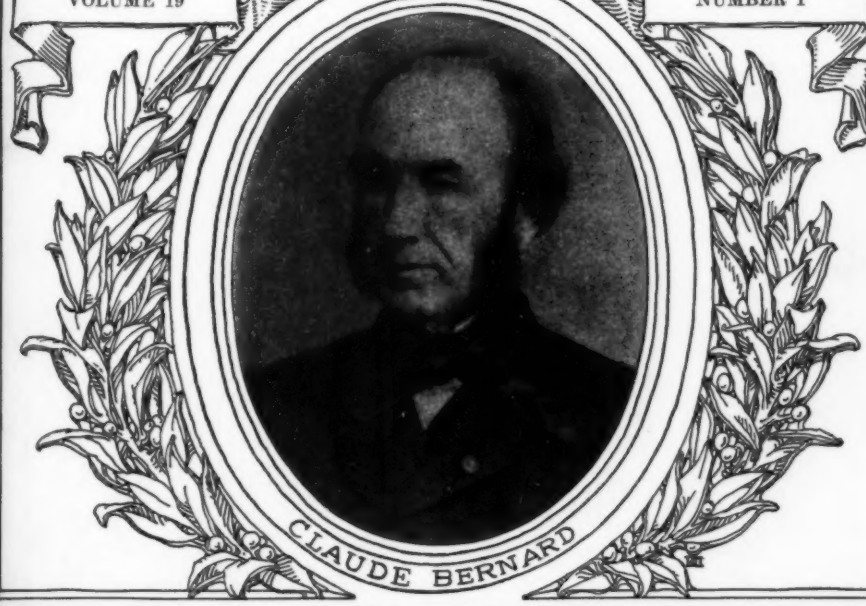


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# 1939 INDEX

## TO AUTHORS' ABSTRACTS

in

• ANATOMY • ANTHROPOLOGY • MORPHOLOGY
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The amounts of the vitamin carriers as given later refer to their dry matter content, and when included in rations they displaced an equal weight of starch. During the early stages of this study each chick received 40 γ of thiamin, and 20 γ of flavin daily by pipette. More recently these vitamins were included in the ration at a level of 200 γ of thiamin and 400 γ of flavin per 100 gm. of ration. The crystalline vitamins were added to the food at intervals of 2 or 3 days.

The key to such success as had been attained by Hogan and Boucher lay in the use of liver extracts, for we have never been able to obtain consistently, in soluble form, from any other source some of the vitamins required by the chick. It was suspected therefore that the later failures mentioned were due to variability in the liver extracts, so in future studies it was decided to rely on extracts prepared in the laboratory. Up to this point the extracts, two in number, had been obtained from commercial sources. A water extract of liver is the starting material in their preparation and this is fractionated with alcohol. One fraction is soluble, the other insoluble in 70% alcohol. Of these two, the soluble fraction seemed the most useful for our purpose, but with some shipments of the commercial products the performance of the chicks was inferior unless both fractions were present in the ration. This indicated that the activity of the extracts was due to more than one component, so the procedure was modified in an attempt to devise a method that would give a more complete separation of the active agents. This attempt has not been entirely successful, but after the change in procedure became effective, the immediate difficulties due to growth failure disappeared. A condensed description of the procedure appears in table 1, and the yields from two representative shipments are reported in table 2.

As a standard of success in our feeding trials two growth curves have been used. One is that published by Heuser and Andrews ('32), and selected because it was the most rapid we have seen for chicks subsisting on practical rations. The other was prepared from our own data for chicks reared on a ration

of natural foodstuffs. In each feeding trial we always run such a control group. This is used as a control to be sure that failures are due to inadequacy of the rations and not to subnormal chicks. As is evident from the graphs, the growth rate on our practical ration is a severe criterion of performance.

TABLE 1

*Plan followed in the preparation of the liver extracts used*

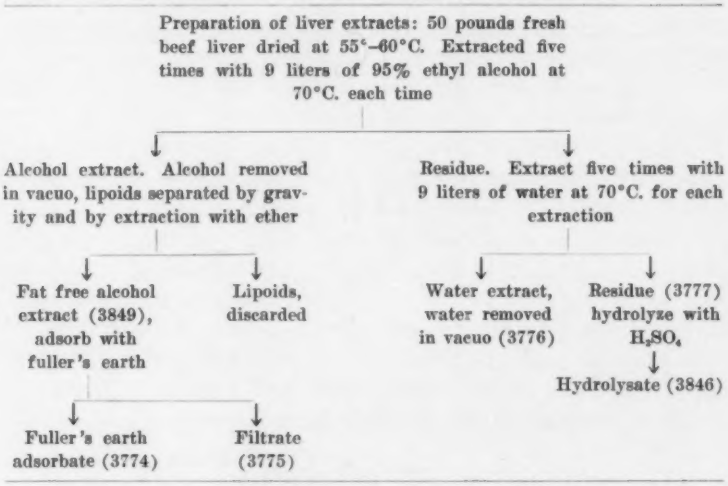
Preparation of liver extracts: 50 pounds fresh beef liver dried at 55°–60°C. Extracted five times with 9 liters of 95% ethyl alcohol at 70°C. each time
Alcohol extract. Alcohol removed in vacuo, lipoids separated by gravity and by extraction with ether
Residue. Extract five times with 9 liters of water at 70°C. for each extraction
Fat free alcohol extract (3849), adsorb with fuller's earth
Lipoids, discarded
Water extract, water removed in vacuo (3776)
Residue (3777) hydrolyze with $H_2SO_4$
Hydrolysate (3846)
Fuller's earth adsorbate (3774)
Filtrate (3775)

TABLE 2

*Yields of liver fractions*

| | FRESH LIVER | DRY LIVER | LABORATORY NO. OF FRACTIONS | | | |
|---|---|---|---|---|---|---|
| | | | 3774 | 3775 | 3776 | 3777 |
| | *gm.* | *gm.* | *gm.*[1] | *gm.* | *gm.* | *gm.* |
| A | 329 | 100 | 1.6 | 2.5 | 6.0 | 55.5 |
| B | 332 | 100 | 2.0 | 3.0 | 7.0 | 63.1 |

[1] The weights as given are of the fuller's earth adsorbate. Only a small fraction was derived from the liver extract.

*Adequacy of liver fractions.* Figure 1 summarizes the rates of growth observed in some of the individual feeding trials. Their reliability has been established, in some cases, by frequent repetition.

Ration 3992 contains both the alcoholic extract and the water extract, and it will be observed that the rate of growth is practically identical with that of the control group. There was one mortality in this group, but it was the only one of the eighteen chicks that received this ration.

As is indicated in table 1, the alcohol soluble material was fractionated by adsorption on fuller's earth, so this adsorbate and the filtrate were combined in the rations of a number of

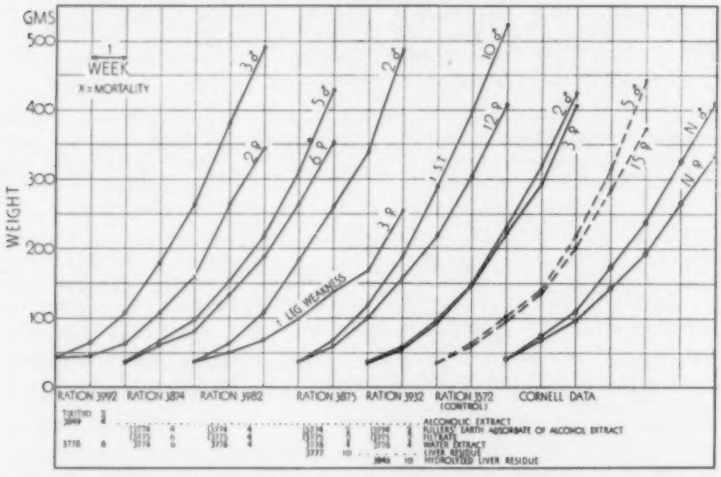


**Fig. 1 The liver fractions here studied contained all of the unrecognized vitamins required by the chick. Rations 3874 and 3992 contained liver extracts only and permitted chicks to grow at the normal rate. The curves for ration 3982 show that in rations of this type 4% of the water-soluble fraction is not enough.**

**Ration 3875 contained the liver residue, and supported a remarkable rate of growth. The hydrolyzed liver residue, present in ration 3932, was of some value but less effective than the residue itself.**

groups. No. 3774 is the fuller's earth adsorbate of the alcohol soluble fraction, no. 3775 is the filtrate, and no. 3776 is the water extract.

When the three soluble fractions were included at the levels shown in ration 3874 the rate of growth was excellent, but when the amounts of preparations 3775 and 3776 were reduced,

as in ration 3982, the response was less satisfactory. In ration 3875 the amounts of the soluble fractions were still further reduced but the thoroughly washed liver residue was included in addition. It is very evident that the liver residue still contained one or more substances which accelerates the rate of growth in a very significant manner. Ration 3932 is very similar to ration 3875, but the acid hydrolyzed liver residue has been substituted for the liver residue. The rate of growth was less rapid, but was still satisfactory when compared with any conventional growth standard. In figure 3 is presented further evidence of the value of the hydrolyzed material.

It is evident that the liver fractions are a satisfactory source of the unrecognized vitamins required by the chick. If supplied in sufficient amount the fractions soluble in alcohol and in water are adequate by themselves. If the amounts of these extracts are much reduced, then it is necessary to include the hydrolyzed liver residue in addition, in order to obtain a normal growth rate.

*Distribution of the required vitamins.* The rations described thus far contained both the alcohol and water soluble fractions. One may ask, therefore, whether both are essential, or helpful. Illustrations of our data on that point are presented in figure 2.

Rations 3881, 3876, and 3877 contained varying amounts of the water soluble fraction, no. 3776, but none of the alcohol soluble fraction. In each instance the growth rate was subnormal. Furthermore the rate of growth was the same, whether the ration contained 10 or 16% of this supplement. Reducing the amount from 10 to 6% of the ration did not greatly reduce the adequacy of the ration. This shows that the water soluble fraction must be supplemented with the alcohol soluble fraction.

Some attention has also been given to the adequacy of the alcohol soluble fraction alone, illustrated by ration 3789 which also contained the liver residue. The growth rate was slow and the mortality was 100%. In ration 3788 the liver residue was omitted and 2% of the water extract was included. The

rate of growth was still below the optimum on this low level, but it is evident that in this type of ration the water extract is indispensable. The essential nature of this component is brought out still more clearly in ration 3887, which supports an extremely rapid rate of growth. Thus ration 3789, a complete failure, was converted into a decided success merely by adding to it 2% of the water soluble fraction.

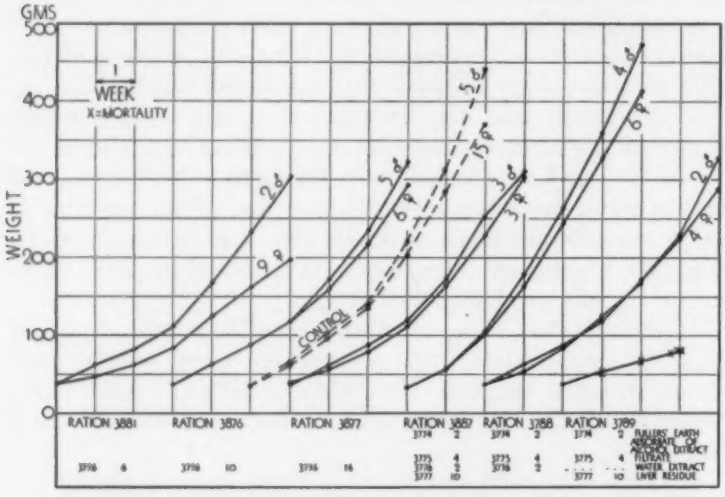


Fig. 2 Rations 3876, 3877, and 3881 contain the water-soluble fraction only, and are unsatisfactory. Ration 3789 contains the alcohol-soluble fraction only and gives a complete failure. Ration 3887 contains both soluble fractions, and is a marked success.

It may be concluded, therefore, that the alcohol extract, although not absolutely indispensable in rations of this type, yet is required in order to secure a high degree of success. The water extract is indispensable, but by itself is inadequate. Presumably a more refined method of preparation should give a sharper separation, and then both the alcohol and water soluble fractions would be found to be absolutely indispensable.

*Nutritional properties of the hydrolyzed liver residue.* How important is the hydrolyzed liver residue in formulating a simplified ration? Figure 3 is presented to show that the acid hydrolyzed liver may contribute something of value, although, as was brought out previously, it is not essential if the other soluble fractions are included in sufficiently large amounts.

Ration 3993 does not contain acid hydrolyzed liver, and chicks which received it grew slowly. When the hydrolyzed product was included, as in ration 3981, the growth rate was markedly accelerated. When, as a further addition, the alcohol soluble fractions were also included (ration 3980), a very

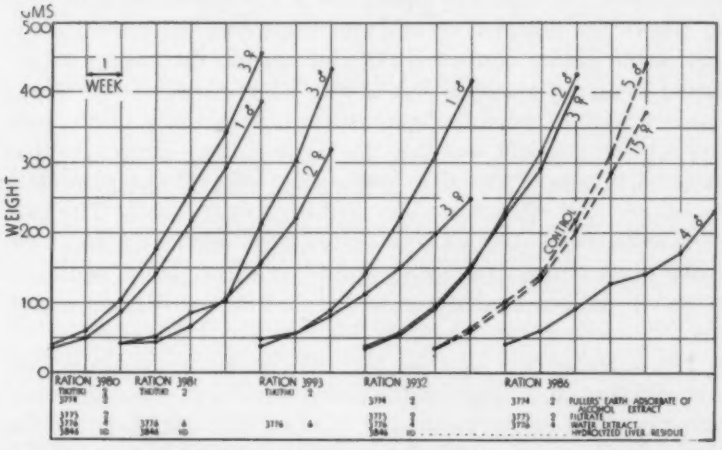


**Fig. 3 Rations which lacked the hydrolyzed residue and contained small amounts of the soluble fractions, such as 3986 and 3993, were unsatisfactory. When the residue was included, as in rations 3980 and 3932, the rate of growth was normal.**

considerable acceleration of growth occurred again. Ration 3986 does not contain acid hydrolyzed liver and is altogether unsatisfactory. When acid hydrolyzed liver was included as in ration 3932 the rate of growth surpasses those that have been described in the literature as normal.

It is concluded, then, that when the alcohol and water extract are supplied at a low level, the ration can be made com-

plete by including the hydrolyzed liver residue. If, however, the alcohol and water extracts are supplied at a sufficiently high level, then the hydrolyzed liver residue does not effect any marked further improvement.

The nature of the active agent present in hydrolyzed liver deserves some comment. We have tentatively assumed it to be a vitamin. However, Arnold, Kline, Elvehjem and Hart ('36) demonstrated that the growth rate of chicks can be definitely improved by the addition of arginine to a natural ration in which corn and wheat proteins are fortified with casein or when casein is the principal protein. Klose, Stokstad and Almquist ('38) reported that if the ration contained only 20% of casein the addition of arginine improved the growth rate, but if the ration contained 30% of casein, then arginine reduced the growth rate if it had any effect at all. Our rations contain 35% of casein so presumably the activity of the acid hydrolyzed product was not due to arginine. However, that possibility was explored by adding 1% of arginine carbonate to rations that did not contain acid hydrolyzed liver. The rate of growth was not at all accelerated, from which we may presume that the active agent in hydrolyzed liver is not arginine. Our data do not enable one to decide whether it is an amino acid, or some other substance liberated by hydrolysis.

*The usefulness of tikitiki.* May tikitiki make any important contribution to a simplified ration for chicks? This material was prepared by the method of Wells ('21). As shown in the previous figures, we have frequently used tikitiki as a carrier of soluble vitamins. The curves presented in figure 4 show that it contains a nutrient which is required by the chick.

Ration 3944 does not contain tikitiki, and is a failure. Ration 3931 is otherwise similar to ration 3944, but it contains 2% of tikitiki and is much superior. Ration 3986 also does not contain tikitiki and, practically speaking, it is a failure. When tikitiki is included though, as in ration 3964, the rate of growth is almost normal. Ration 3932 contains all of the liver fractions and no tikitiki, and is a complete success, which indicates that the alcohol soluble fraction contains the same factors as

does tikitiki. This is shown still more clearly in ration 3980 which contains 2% of tikitiki. However, chicks do not grow more rapidly on this ration than on ration 3932 which contains no tikitiki. It may be concluded then, that if the alcohol soluble fraction is present in liberal amounts, tikitiki may be omitted. Usually, however, chicks grow more rapidly when it is included.

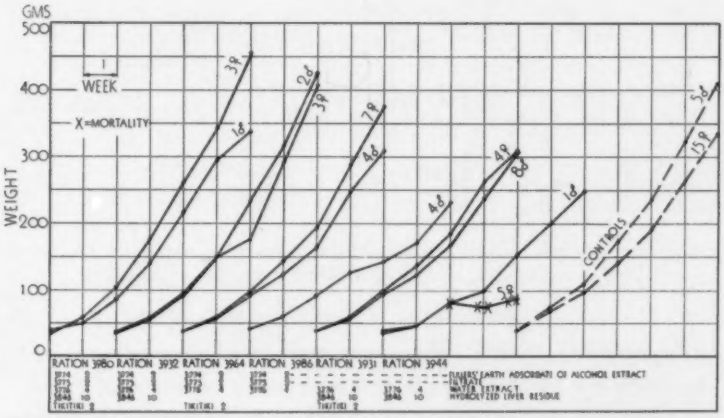


**Fig. 4 Ration 3944 does not contain tikitiki and is a failure. The addition of tikitiki, as in ration 3931, brings about marked improvement. Ration 3986 lacks tikitiki and is unsatisfactory, but when tikitiki is included as in ration 3964, the growth rate is greatly accelerated. Ration 3932 contains the alcoholic extract and it is not improved when further fortified with tikitiki, as in ration 3980.**

The mortality rate is highly significant in an assay of the adequacy of a ration, and therefore a record of the deaths is summarized in table 3. Only the chicks on rations that offered some promise were considered in the preparation of this table, although it should be mentioned that some of the rations are not described in this report. The chicks used for these calculations are distributed among three groups, depending on whether their ration contained the liver residue, the hydrolyzed liver residue, or neither of these constituents.

None of the chicks was obviously defective when placed under observation, although some were smaller and weaker

than others. Furthermore, there was no weeding out after the feeding trials started, so the total mortality was satisfactorily low under the circumstances. A total of 290 chicks is included in table 3, and only three, slightly over 1%, died after they were 2 weeks old. It is evident, then, that there are no unusual difficulties in rearing chicks on these simplified rations.

TABLE 3

*Mortality rate*

| RATION CONTAINS | NUMBER OF CHICKS | TOTAL MORTALITIES | DEATHS DURING | | |
|---|---|---|---|---|---|
| | | | 1st week | 2nd week | 3rd–6th week |
| | | % | % | % | % |
| Liver residue | 114 | 3.5 | 0 | 1.75 | 1.75 |
| Hydrolyzed liver residue | 79 | 8.8 | 3.5 | 5.3 | 0 |
| Soluble extractives only | 97 | 6.2 | 4.1 | 1.1 | 1.0 |

## SUMMARY

1. If recognized vitamins, i.e., A, $B_1$, $B_2$ and D, are otherwise provided, then all the unrecognized vitamins required by the chick are present in alcohol and water extracts of liver.

2. Partial separation of the unidentified essential nutrients can be attained by extracting first with alcohol and then with water. Both extracts must be present if the ration is to be adequate.

3. When the alcohol and water extracts were supplied at a low level, then the liver residue, or hydrolyzed liver residue, supplied some essential nutrient. If, however, the extracts were supplied at a sufficiently high level, then there was no further increase in the effectiveness of the ration when the hydrolyzed product was included.

4. Tikitiki contains an essential nutrient, but the data thus far available indicate that this same nutrient is present in the alcohol soluble fraction, and presumably in even greater concentration.

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# ADEQUACY OF A MILK DIET FOR THE RAT [1]

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THREE FIGURES

(Received for publication July 17, 1939)

The major nutritional deficiencies of milk are well known, but even after these are supplied it is still questionable whether this food, as the sole article of diet over long periods of time, will maintain the optimum nutritional state. Of the various investigators who have attacked this problem, Daniels and Everson ('35) were probably the most successful, and their paper should be consulted for a review of the earlier literature. These workers were primarily interested in another problem, however, and used too few animals, over periods that were too short for a precise assay of the adequacy of a food. The tests were conducted with rats and the third generation consisted of four females, all of which produced first litters and three produced second litters. The fourth generation consisted of five females and the experimental period ended with their first litters.

At about this same time an investigation was under way in this institution which required that female rats be maintained throughout the greater part of their reproductive periods on a milk diet. Since fluid milk is too dilute to permit lactating rats to consume the optimum amount of energy, it was fortified by the addition of whole milk powder. The number of litters produced was far below that expected, and when the original investigation was completed we took charge of the female progeny in an attempt to discover the nature of the deficiency.

[1] Contribution from the Missouri Agricultural Experiment Station journal series no. 617.

## EXPERIMENTAL

The initial animals used in this investigation had received from the time they were weaned a milk diet only, consisting of equal parts by weight of fresh unpasteurized milk and whole milk powder.[2] This was supplemented with 2 cc. of mineral mixture 2089 (table 1) per 100 gm. of the milk preparation. The average amount of the milk mixture consumed was 25 cc. per rat daily, and it was estimated that this amount of food contained 2.5 mg. of added iron, 0.254 mg. copper, 0.167 mg. manganese, and 0.076 mg. of iodine. The milk was from pure

TABLE 1

*Composition of mineral mixtures*

| COMPOUND | WEIGHT | | ELEMENT | WEIGHT OF ELEMENT PER RAT DAILY | |
|---|---|---|---|---|---|
| | 2089 [1] | 2089A [2] | | 2089 | 2089A |
| | *gm.* | *gm.* | | *mg.*[3] | *mg.* |
| $FeSO_4 \cdot 7H_2O$ | 5.00 | 5.00 | Fe | 2.5 | 5.00 |
| $CuSO_4 \cdot 5H_2O$ | 0.40 | 0.63 | Cu | 0.254 | 0.80 |
| $MnSO_4 \cdot 4H_2O$ | 0.27 | 0.82 | Mn | 0.167 | 1.02 |
| KI | 0.04 | 0.04 | I | 0.076 | 0.15 |
| HCl conc. | 1.0 cc. | 1.0 cc. | | | |
| $H_2O$ | 200.0 cc. | 200.0 cc. | | | |

[1] Two cubic centimeters per 100 gm. of ration.

[2] One cubic centimeter per rat per day.

[3] Estimated from daily food intake.

bred Holsteins. During the growing season the cows, except for the dry summer of 1936, had adequate pasture of lespedeza, sweet clover, or Sudan grass. No information on the previous litter records was available beyond the statement that few litters had been produced, at very irregular intervals.

The animals were maintained on this same ration for some weeks, but in conformity with their previous history their reproductive activity continued at a very low level. It was suggested that the limiting factor was one of the known vitamins, and the milk ration just described was changed to include the more commonly used vitamin carriers. This new ration contained 50 parts of fresh unpasteurized milk,

[2] Klim, obtained from the Borden Company, New York.

and 50 parts of a mixture made up of 88.5% of whole milk powder, 10% of dried yeast, 0.5% of cod liver oil, and 1.0% of wheat germ oil. The mineral supplement was unchanged. This modification did not improve the reproductive performance and after a time the inclusion of yeast, cod liver oil, and wheat germ oil was discontinued. These females were then discarded and the observations continued with their daughters, designated as the first generation. They were weaned when 28 days old and distributed in groups of three or four in metal cages provided with screen floors of ½ inch mesh. Their daily intake of food, and of minerals (mixture 2089) was approximately the same as that of their mothers. The freshly mixed diet, in glass or porcelain containers, was supplied each morning and evening. These experimental periods began in December, 1934, and in the following January.

When the females had attained weights of 175 to 200 gm. they were mated with a male from the stock colony. They were weighed weekly and when they became pregnant, as shown by a marked gain in weight, were placed in individual cages. The young were weaned when they were 28 days old and the mothers returned to the breeding cage without a rest period. Females on the milk ration were not discarded until they had failed to produce a litter for an average of 90 days.

The controls were females in the stock colony which were maintained on Steenbock's stock diet ('23). Each of them received 25 cc. of milk daily except when lactating and then the amount was increased to 35 cc. When these weaned their litters they, too, were returned at once to the breeding cage, with no rest period. This no doubt accounts in large measure for the high infant mortality in both groups. These animals were discarded if they did not produce a litter within 60 to 70 days after they were returned to the breeding cage. These rats are not classified by generations, either in the text or in the figures. They and their ancestors had received this same ration for approximately 15 years, and over long periods of time the reproductive rate does not materially change.

In the meantime the report of Daniels and Everson ('35) appeared which suggested that their ration may have been superior to the one we were using. Since the milk diet they used contained considerably larger quantities of the added minerals our ration was changed in July, 1935. From that time on 1.0 cc. of mixture 2089A, described in table 1, was supplied to each animal daily. The mineral solution was placed in the food container each morning with a small amount of milk and when that was consumed, usually in an hour or 2, the remainder of the allowance of milk was supplied. Furthermore, since the consistency of the milk mixture previously used was unsatisfactory, the proportions were changed to 500 cc. of fresh milk and 250 gm. of whole milk powder. The amounts consumed were not recorded.

The improved performance of the rats after the ration was modified seemed to justify the change. Eight females were mated 90 days before the change was made, and in that time they produced an average of 0.5 litter per animal. In the 90 days after the change was made these same rats gave birth to an average of 1.1 litters per animal. It is well known that the initial productivity of rats is normally higher than was observed in these rats. For example, their fourth generation descendants bore an average of over 1.4 litters per animal in a comparable period. Twenty-three colony rats were mated at about the same time as were the original eight experimental females, and during the first 90 days they bore an average of almost 1.3 litters per animal.

## RESULTS

The litter and weaning records of four generations on the experimental ration, and of all animals on the control ration are summarized in figures 1, 2 and 3. Figure 1 shows that at birth the litters of the control animals were significantly larger than those of the experimental group, and that they were still slightly larger at weaning. Also, among the experimental animals there was a tendency in successive generations for the litters to become larger, both at birth and at weaning. It will

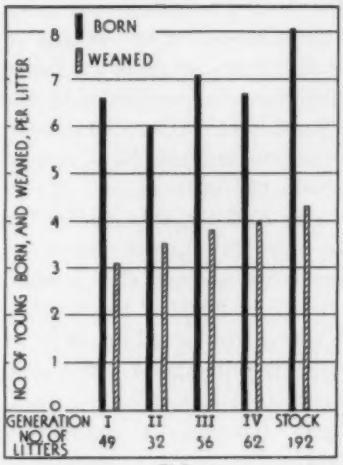


FIG. 1

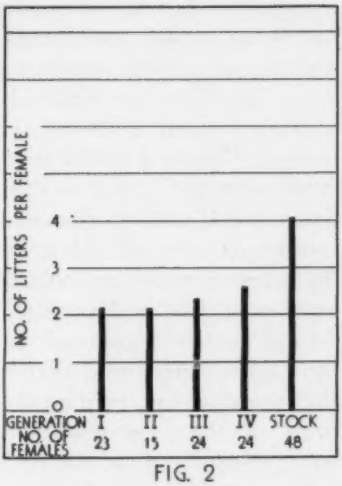


FIG. 2

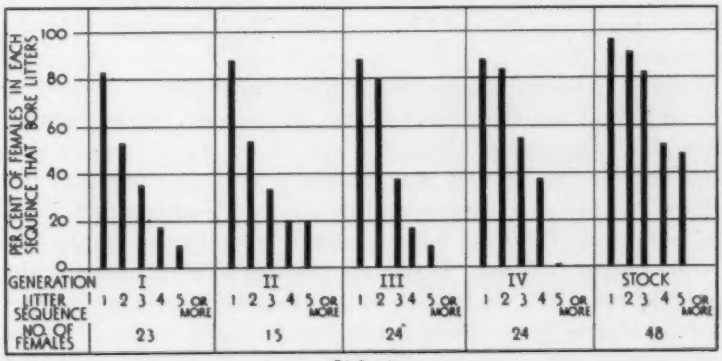


FIG. 3

**Fig. 1** Females which received the mineralized milk diet did not bear or wean as many young per litter as did the controls.

**Fig. 2** Females which received the mineralized milk diet failed to bear a normal number of litters.

**Fig. 3** The ordinate shows the percentage of females in each generation sequence that bore 1, 2, 3, 4 and 5 or more litters. All experimental females produced almost the normal number of first, and the third and fourth generation produced almost the normal number of second litters. In all generations the number of third, fourth and fifth litters was abnormally low.

be recalled that the females of the first generation had been restricted for the early part of their lives to a low intake of manganese, and this may account for their inferior performance. We have no explanation for the observation that the number weaned by the third and fourth generations continued to rise. Figure 2 shows that the control animals bore almost twice as many litters as did those on the milk ration. From figure 3 it is evident that most of this difference is due to the smaller number of third, fourth, and fifth litters borne by the experimental animals. They bore almost the normal number of first and second litters. Only 20% of the experimental females produced five or more litters, while 48% of the controls produced that number. It will also be observed that there was no evident deterioration in succeeding generations of the experimental animals, the fourth generation being about as successful as the first.

Since the increase in the amount of minerals, especially of manganese, was followed by such notable improvement in the number of litters produced, one may ask if another increase would have been followed by further improvement. According to our analysis the solids in the Steenbock ration contain 0.00172% manganese. Peterson and Skinner ('31) report that the average amount of manganese in milk is 0.028 mg. per liter. If, then, a rat consumed 20 gm. of the solids and 35 cc. of milk daily the total daily intake of manganese would still be less than 0.35 mg. In all probability this estimate is decidedly too high. Inasmuch as the experimental animals received almost three times as much manganese as the controls, one would not expect further enrichment of the milk diet with respect to this element to be helpful.

Some additional details are shown in table 2. Even with our system of mating, it would be impossible for a female to produce a litter much oftener than every 56 days. The interval among the control animals was 65.5 days, and among the experimental animals it was approximately 100 days. The complete data would lead one to expect that under the same conditions this rate of performance would continue for

some time at least. The ration we used is still deficient though in one or more substances which must be supplied if the females are to bear a normal number of litters.

TABLE 2

*Length of experimental period and frequency of litters*

| GENERATION | LENGTH OF EXPERIMENTAL PERIOD | | TOTAL NUMBER LITTERS BORN | AVERAGE INTERVAL BETWEEN LITTERS |
|---|---|---|---|---|
| | Average | Range | | |
| | *days* | *days* | | *days* |
| First | 218 | 124–439 | 49 | 102 |
| Second | 220 | 70–461 | 32 | 103 |
| Third | 257 | 70–433 | 56 | 110.4 |
| Fourth | 247 | 133–322 | 62 | 95.8 |
| Stock | 262 | 70–428 | 192 | 65.5 |

## SUMMARY

1. Four generations of female rats which received milk supplemented with iron, copper, manganese, and iodine, produced and weaned almost as many young per litter as did the controls.

2. The experimental animals produced about half as many litters per female as did the controls.

3. Apparently milk is deficient in some nutrient other than iron, copper, manganese, or iodine, which is essential for normal reproduction.

4. The reproductive performance of the fourth generation was not inferior to that of any of the preceding three.

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# BIOLOGICAL ASSAY OF THIAMIN WITH CHICKS

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TWO FIGURES

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Biological assay of thiamin is commonly made with rats. The recent results of Miller ('39), for example, demonstrate that the rat is a highly sensitive test animal in its response to thiamin. The chick is also known to be very sensitive to thiamin deficiency, and a method for bio-assay of thiamin with chicks was described by Arnold and Elvehjem ('38), who reviewed the earlier literature. The present communication describes a method for the assay of thiamin with chicks in which the test diet is evaluated by comparison with a response curve derived by plotting results obtained with graded levels of thiamin. By means of this, the thiamin content of test diets which fail to afford complete protection against polyneuritis may be estimated by measurement of the degree of postponement of polyneuritic symptoms. The use of polyneuritic symptoms, rather than growth rate, for assay purposes has the obvious advantage of specificity.

The problem of supplying all other members of the vitamin B complex in a form free from thiamin has still to be solved. Autoclaved supplements are frequently used for this purpose. Autoclaving has been demonstrated (Jukes, '39) to have a destructive effect upon the filtrate (chick anti-dermatitis) factor (pantothenic acid). However, it was found possible to autoclave yeast to an extent which apparently destroyed the thiamin almost completely, but which permitted the retention of an appreciable proportion of the filtrate factor content.

## EXPERIMENTAL

### *A. Preliminary experiments with an autoclaved diet*

In the method of Kline and co-workers ('32–'33), the entire cereal and casein portion of the diet is subjected to autoclaving. Experiments were made with a diet similar to that described by Kline and co-workers. It was found that polyneuritis was readily produced on the basal diet, and that polyneuritic symptoms could be prevented by addition of a fullers' earth adsorbate, containing thiamin, to the basal diet. However, when the amount of fullers' earth adsorbate was increased threefold over the level which just protected against polyneuritis, growth was very slow. The chicks developed a crow-like appearance, and their crops became enlarged and hematomatous. Autoclaving is known to reduce the biological value of proteins in cereal grains (Morgan and King, '26). The basal diet of Kline and co-workers was later modified by Arnold and Elvehjem ('38) to contain 2% of untreated vacuum-desiccated whole liver substance. Subsequent investigations in the Wisconsin laboratory placed the thiamin content of dried beef liver at 12 micrograms per gram (Mickelsen et al., '39).

### *B. Use of an unheated diet supplemented with autoclaved yeast*

The diet of Almquist and Stokstad ('35) is known to produce rapid growth in chicks when supplemented with vitamin K to prevent fatal hemorrhages (Almquist, '37). The only source of appreciable amounts of thiamin in this diet is yeast. Autoclaving the yeast should make the diet deficient in thiamin. It was found that autoclaving the yeast used in this investigation at 120°C. for 4 hours destroyed 75% of its filtrate factor content, leaving 6 units per gram. Fifteen per cent of autoclaved yeast was hence included in the basal diet. The basal diet had the following composition (diet 152):

| | PARTS |
|---|---|
| Ground polished rice | 68 |
| Washed sardine meal[1] | 15 |
| Autoclaved dried brewers' yeast | 15 |
| Ground limestone | 1 |
| Salt | 0.5 |
| Manganous sulfate | 0.05 |
| Fish oil blend containing 3000 units of vitamin A and 400 units of vitamin D per gram | 0.3 |
| Hexane extract of alfalfa meal, equivalent to one part of alfalfa meal, evaporated on the diet | |

Polished rice supplies small amounts of thiamin (Williams and Spies, '38, p. 238). However, it is not possible to obtain satisfactory growth when polished rice is replaced by purified foodstuffs in a diet of this type when fed to chicks. Evidently polished rice supplies a nutritional essential not found to any appreciable extent in yeast (Hogan, Guerrant and Kempster, '25; McFarlane, Graham and Hall, '31). Confirmation of these earlier observations was made in the present investigation.

Synthetic thiamin chloride hydrochloride[2] (abbreviated subsequently to 'thiamin') was dried to constant weight in a vacuum desiccator over sulfuric acid. Solutions were prepared by dissolving 20 mg. of thiamin in 100 cc. of 20% ethanol containing 1 milliequivalent of hydrochloric acid. Aliquots of the solution were mixed in the test diets and allowed to evaporate at room temperature. Ninety-five per cent ethanol was frequently used as a diluent to aid in the mixing process.

Single-Comb White Leghorn chicks were removed from the incubator on the day following the twenty-first day of incubation, placed in electrically heated battery brooders, and fed the experimental diets immediately. From ten to fifteen chicks were used in a group. Growth and food consumption records

[1] Prepared by washing sardine meal repeatedly with hot water and drying in a large vacuum oven. Kindly supplied by Dr. E. L. R. Stokstad, Western Condensing Company, Petaluma, California. Washed casein may be substituted for washed sardine meal.

[2] Generously furnished by the Merck Institute, Rahway, New Jersey.

were kept. The assay period lasted for 4 weeks. Chicks develop thiamin deficiency so rapidly that no depletion period on the basal diet is needed.

Symptoms of polyneuritis usually first appeared on the ninth or tenth day in chicks on the basal diet, and the birds rarely lived beyond the thirteenth day. Addition of graded levels of thiamin to the diet resulted in the postponement or disappearance of the incidence of symptoms until, at a level of 100 micrograms of added thiamin per 100 gm. of diet, protection was usually complete during the 4-week assay period. Individual variation was so marked that it was considered advisable to use at least ten chicks in each test group. Birds were closely watched for symptoms of polyneuritis, characterized by opisthotonos and inability to stand. Death usually followed within 1 or 2 days. Loss of weight always preceded the appearance of polyneuritic symptoms. Some birds died, often during the night, without polyneuritic symptoms being noted. Such birds were eliminated from calculation. The birds dying with polyneuritic symptoms, together with birds surviving the assay period, were used to calculate the 'polyneuritic mortality index' of the test group. The index value of a bird was calculated by subtracting the number of days it survived from the number of days (28) in the assay period. These values were averaged to give the index value of the test group. Table 1 illustrates the method of calculation. The index value of the group is obtained by adding the figures in the last row and dividing by the number of chicks (12) to give a value of 3.2.

A diet which gave complete protection thus gave an index value of zero, and the index value of the basal diet was usually between 16 and 17. In the fall months this value may rise to about 19, with corresponding increases in the index values of supplemented diets. The indication is that there was less thiamin deposited in the egg during the fall months, since the diet of the parent hens was unchanged. Table 2 summarizes a number of the index values and figure 1 illustrates the response curve obtained by plotting index values against the

TABLE 1

*Illustration of the method of calculation. Group no. 152-28. Supplement = 70 micrograms thiamin per 100 gm. diet. Assay period = 28 days*

| Bird no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age at which polyneuritic symptoms first observed (days) | 21 | 16 | a | a | a | a | 23 | 27 | 15 | a | 20 | a | a |
| Age at death (days) | 22 | 17 | b | b | b | b | 26 | b | 15 | b | 22 | b | 8 |
| Number of days of survival subtracted from number of days in assay period [c] | 6 | 11 | 0 | 0 | 0 | 0 | 2 | 0 | 13 | 0 | 6 | 0 | d |

a Polyneuritic symptoms not observed.

b Survived the assay period.

c This value is the 'polyneuritic mortality index.' Birds surviving the assay period receive a value of zero, indicating complete protection.

d Eliminated from calculation, since death was not observed to be preceded by polyneuritic symptoms.

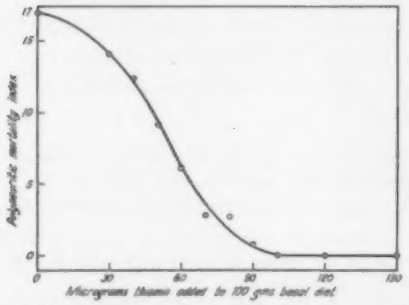
The index value of the group is obtained by adding the figures in the last row and dividing by the number of chicks (12) to give a value of 3.2.

TABLE 2

*Polyneuritic mortality indices obtained with varying levels of thiamin*

| AMOUNT OF THIAMIN ADDED TO 100 GM. OF BASAL DIET 152 | POLYNEURITIC MORTALITY INDICES | | | | | | | TOTAL NUMBER OF CHICKS INVOLVED |
|---|---|---|---|---|---|---|---|---|
| micrograms | Expt. 1 | 2 | 3 | 4 | 5 | 6 | Average | |
| 0 | 16.0 | 16.8 | 17.0 | 16.7 | 17.3 | 18.0 | 17.0 | 77 |
| 30 | | | | 12.3 | 15.9 | | 14.1 | 30 |
| 40 | | | | 10.7 | 14.2 | | 12.4 | 30 |
| 50 | 7.9 | | 10.5 | 6.5 | 12.1 | 9.1 | 9.2 | 64 |
| 60 | | 3.0 | | | 9.2 | | 6.1 | 25 |
| 70 | 1.8 | | 3.2 | | | 3.4 | 2.8 | 34 |
| 80 | | 2.7 | | | | | 2.7 | 10 |
| 90 | | | 0 | | 1.5 | | 0.8 | 27 |
| 100 | 0 | 0 | | | | | 0 | 22 |
| 120 | | 0 | 0 | | | 0 | 0 | 37 |
| 150 | 0 | | | | | | 0 | 12 |

level of thiamin added to the diet. It will be seen that the method reaches its maximal sensitivity in the region between 30 and 70 micrograms. In conducting an assay, therefore, an amount of the test material expected to furnish an increase of about 50 micrograms of thiamin is added to 100 gm. of the basal diet. In this region the average food consumption per bird per day is about 5 gm. and a difference of 10 micrograms of thiamin per 100 gm. of diet is sufficient to produce a definite change in the index. This difference corresponds to an intake of less than 1 microgram of thiamin per bird per day.



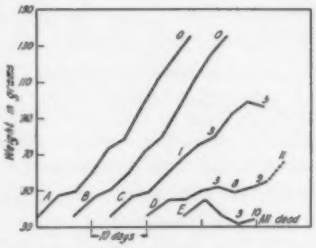




Fig. 1 Response curve obtained by plotting average polyneuritic mortality indices, from table 1, against levels of thiamin added to diet 152.

Fig. 2 Growth curves of chicks in experiment 1. Twelve chicks were used in each group. The thiamin supplement added to 100 gm. of basal diet was for group A, 150 micrograms; B, 100 micrograms; C, 70 micrograms; D, 50 micrograms; E, none (basal diet). The numbers above the curves represent the cumulative total of observed cases of polyneuritis. The last part of the growth curve of group D is represented by a broken line because only two birds survived after the eleventh day. All the chicks in group E were dead by the fourteenth day.

### *C. Growth and food consumption*

Figure 2 illustrates the growth in experiment 1. The average daily food consumption per bird, obtained by dividing the total food consumption by the sum of the number of days of survival of each chick, was for group A, 11.8 gm.; group B, 11.7 gm.; group C, 6.9 gm.; group D, 3.8 gm.; group E, 1.0 gm. Part of the diet was placed on the floor of the cage on sheets of paper for the first 4 days to encourage the chicks to start

eating, and hence the first 4 days were eliminated from calculation of food consumption due to wastage of diet. The food consumption data well illustrate the marked effect of thiamin deficiency upon appetite. Figure 2 shows that the growth of chicks was not improved by increasing the level of thiamin in the diet beyond the level (100 micrograms) which just protected against polyneuritis. This conclusion was supported by the results of the other experiments.

TABLE 3

*Assay of polished rice for thiamin content*

| AMOUNT OF THIAMIN ADDED TO 100 GM. OF GLUCOSE DIET | POLYNEURITIC MORTALITY INDEX: EXPERIMENT | | THIAMIN ADDITION TO RICE DIET CORRESPONDING TO THE PRECEDING INDEX, OBTAINED FROM FIGURE 1 | DIFFERENCE BETWEEN GLUCOSE AND RICE DIETS, IN TERMS OF THIAMIN PER 100 GM. OF DIET |
|---|---|---|---|---|
| micrograms | 1 | 2 | micrograms | micrograms |
| 0 | | 21.6 | a | |
| 30 | 17.1 | | 0 | 30 |
| 40 | 15.2 | | 23 | 17 |
| 50 | 14.0 | | 30 | 20 |
| 70 | | 11.2 | 43 | 27 |
| 90 | | 7.3 | 57 | 33 |
| 120 | 2.3 | 1.9 | 79 [b] | 41 |
| 150 | | 0 | Complete protection | .. |

a Beyond lower limit of figure 1.

b From the average value of the two indices.

### *D. Estimation of thiamin content of polished rice*

Glucose [3] was substituted for polished rice in the basal diet, and various levels of thiamin were added. The index values obtained were compared with the response curve (fig. 1) with the results shown in table 3.

The average value of the difference between the glucose and rice diets is 28 micrograms per 100 gm. of diet. Assuming that the glucose was free from thiamin, it may be deduced that 68 gm. of polished rice contained 28 micrograms of thiamin, corresponding to 0.4 micrograms of thiamin per gram.

[3] 'Cerelose.'

Growth was slow on the glucose diet even when completely supplemented with thiamin. The chicks in the group receiving an addition of 150 micrograms per 100 gm. of diet averaged only 63 gm. in weight at 28 days of age. Groups of chicks which received the rice diet completely supplemented with thiamin weighed from 110 to 134 gm. at 28 days. This result may be compared with the observations of Hogan, Guerrant and Kempster ('25) who found that chicks receiving a ration composed of starch, casein, salt mixture, yeast and cod liver oil grew slowly, but that growth was markedly accelerated when the corn starch was replaced by polished rice. Similar results were noted by McFarlane, Graham and Hall ('31) and by Stokstad and Manning ('39). For this reason, even though the glucose diet was more deficient in thiamin, it was thought desirable to use the rice diet for regular assay purposes. Substitutions for assay purposes may be made in the rice diet with the assumption that polished rice supplies 0.4 micrograms of thiamin per gram. Since an addition of 100 micrograms was just sufficient to protect the birds on diet 152, the minimal thiamin requirement of the chick was at least 128 micrograms per 100 gm. of diet for the first 4 weeks under the experimental conditions involved, basal diet 152 supplying at least 28 micrograms of thiamin per 100 gm. due to its content of polished rice. The amount of thiamin supplied by other constituents of the diet is presumably quite small, and the minimal thiamin requirement of the chick may probably be estimated as between 130 and 150 micrograms per 100 gm. of diet.

## DISCUSSION

The response curve illustrated in figure 1 appeared to be fairly constant for the chicks used in the present investigation. Others who use the method may wish to construct response curves under their own conditions. For this purpose, it is suggested that in making an assay the following supplements should be fed to test groups: group 1, none (basal diet); group 2, 30 micrograms; group 3, 50 micrograms; group 4,

70 micrograms; group 5, 100 micrograms; group 6, 150 micrograms of thiamin per 100 gm. of diet; group 7, enough test substance to furnish about 50 micrograms; group 8, enough to furnish about 100 micrograms per 100 gm. of diet. Groups 2 and 6 are less important than the other groups. Substitutions may be made for a part of the polished rice, assuming a content of 0.4 micrograms of thiamin per gram for this material in calculating the results. If the material to be tested is high in protein, part of the fish meal in the basal diet may be replaced. In the case of materials unusually low in thiamin, it may not be possible, of course, to supplement the diet with levels of the material furnishing as much as 50 micrograms of thiamin per 100 gm. of diet. In such a case, it is necessary and feasible to use the upper portion of the response curve in calculating the results.

The gregarious eating habits of the chick are advantageous in biological assays in that a group of chicks will usually feed in comparative unison. Food spillage is small with suitably constructed troughs. It is particularly desirable to examine the chicks for symptoms of polyneuritis in the late evening during the final 3 weeks, since cases frequently develop during the evening and terminate fatally before the next morning.

The method described in the present communication has been used in the assay of muscular tissue from pigs fed diets containing various levels of thiamin (Hughes and Heitman)[4], in a study of thiamin in the diets of ruminants (McElroy and Goss, '39) and in the assay of commercial dog food for thiamin (Caldwell).[5]

## SUMMARY

1. A method is described for the assay of thiamin with chicks, using a basal diet consisting principally of polished rice, fish meal and autoclaved yeast.

2. A response curve was constructed in which the level of thiamin added to the diet is compared with the 'polyneuritic mortality index,' the latter value being expressive of the de-

[4] To be published.

[5] Personal communication.

gree of protection afforded by a suboptimal level of thiamin fed during the assay period of 28 days.

3. The thiamin requirement of the chick was estimated as being between 130 and 150 micrograms of thiamin chloride hydrochloride per 100 gm. of diet for prevention of polyneuritis under the conditions studied.

4. The thiamin content of the polished rice used in the basal diet was found to be about 0.4 micrograms per gram. A growth essential for the chick, absent from autoclaved yeast, was found present in polished rice.

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# PREVENTION OF HYPERPLASIA IN THE FORESTOMACH EPITHELIUM OF RATS FED WHITE FLOUR

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Hyperplasia of the squamous epithelium in the forestomach of rats has been the subject of numerous studies since Pappenheimer and Larimore ('24) found the lesions in rats fed a diet of white flour. The similarity of the papillomatous changes to the non-malignant type produced by Fibiger ('19) with Spiroptera was recognized and they suggested that an irritating substance was ingested. It was found that the rats were plucking and eating their hair and that hairs could be found embedded in the abnormal epithelium. However, ingestion of hair by normal animals did not produce the changes, which led Pappenheimer and Larimore to conclude that an essential dietary factor was missing in addition to the presence of an irritating substance. Recent dietary studies (Passey, Leese and Knox, '35) suggest that the lesions obtained in rats and mice by Fibiger depended to a large extent upon his diet of white bread. There is now general agreement that the lesions are induced by an incomplete diet but there is no agreement on the nature of the dietary fault. A number of authors suggest that vitamin A deficiency produces the lesions and quote the work of Fujimaki ('26). Yet, Fujimaki, who first reported the effect of vitamin A deficiency, found later ('31) that it was unimportant. Howes and Vivier ('36) demonstrated that yeast will prevent the lesions but, in contrast to the studies of Hoelzel and DaCosta ('37) who ascribe the lesions to a deficiency of biologically good protein,

found that a diet fortified with 20% casein will not provide protection. Findlay ('28) produced the lesions with vitamin $B_2$ deficiency but not with $B_1$ deficiency.

In previous studies (Sharpless, '37) with a purified low-protein diet, extreme hyperplasia occurred which was prevented by feeding cystine. However, the need for further study was evident when cystine failed to prevent the lesions in rats fed the white flour diet.

## EXPERIMENTAL PART

The general plan of the experiment was to study, in rats fed a white flour diet, the effect of cystine and the vitamins known to be in yeast upon the development of gastric lesions. Two preliminary experiments showed that a deficiency in the protein of white flour was not the causative factor. First, the white bread flour diet fortified with 10% of vitamin free casein did not prevent the lesions, which confirmed the same finding of Howes and Vivier ('36). Second, fortification with 10% of gelatin together with the necessary amino acids to make it a complete protein did not prevent the lesions.

Each experiment in the present series extended over a 6-week period. In all cases young rats, both male and female, weighing (with few exceptions) between 60 and 80 gm., were kept in wire cages and were provided with food and water ad lib. Except for the addition of 5% butterfat and viosterol, the basal diet was the same as that used by Pappenheimer and Larimore ('24). Litter mate controls were fed the basal diet in each experiment.

The following concentrates, in all possible combinations, were added to the basal diet: 'Labco' desiccated rice polish concentrate, lactoflavine, nicotinic acid, and cystine. The composition of the diets is given in table 1.

## RESULTS

None of the diets which contained only one concentrate prevented the lesions, whereas the diet which included all of them gave almost complete protection. The results are

recorded in table 2. The lesions have been well described by Pappenheimer and Larimore ('24) and also by Howes and Vivier ('36). All animals with one or more hyperplastic areas visible to the naked eye were recorded as having lesions. No attempt was made to record in the table differences in severity of the lesions.

Of twenty-five rats fed diet 229, which contained all four supplements, just one had a papillomatous area and it was

TABLE 1

*Composition of diets*[1]

| DIET NO. | WHITE BREAD FLOUR | RICE POLISH CONCENTRATE | LACTO-FLAVINE | CYSTINE | NICOTINIC ACID | VITAMIN $B_1$ |
|---|---|---|---|---|---|---|
| | % | % | μg.% | % | % | % |
| 151 | 90.0 | ... | ... | ... | ... | |
| 152 | 90.0 | ... | 100 | ... | ... | |
| 154 | 89.0 | 1.0 | ... | ... | ... | |
| 175 | 88.0 | 2.0 | 200 | ... | ... | |
| 177 | 87.5 | 2.0 | 200 | 0.5 | ... | |
| 178 | 87.5 | 2.0 | ... | 0.5 | ... | |
| 190 | 89.5 | ... | 100 | 0.5 | ... | |
| 191 | 89.5 | ... | ... | 0.5 | ... | |
| 199 | 89.0 | ... | ... | ... | 1.0 | |
| 229 | 88.0 | 1.0 | 100 | 0.5 | 0.5 | .... |
| 236 | 89.0 | ... | ... | 0.5 | 0.5 | .... |
| 237 | 89.0 | ... | 100 | 0.5 | 0.5 | .... |
| 238 | 88.0 | 1.0 | ... | 0.5 | 0.5 | .... |
| 239 | 88.5 | 1.0 | ... | ... | 0.5 | .... |
| 240 | 89.5 | ... | 100 | ... | 0.5 | .... |
| 251 | 88.5 | 1.0 | 100 | ... | 0.5 | .... |
| 269 | 89.0 | ... | 100 | 0.5 | 0.5 | 0.002 |

[1] Each diet contained in addition, 5% butterfat, 2.9% calcium lactate, 2% sodium chloride, 0.1% ferric citrate and 15 drops of viosterol per kilo.

very small. This diet was the only one which provided such complete protection although diet 251 which contained all of the supplements but cystine produced one or two small papillomatous areas in each of five rats from a group of nineteen. Diet 154, where the supplement was rice polish concentrate, suggests some protection, but the controls did not show a high incidence of lesions and a diet containing 2% of the concentrate provided no greater protection than

that obtained from 1%. The purpose of the experiment was to obtain complete protection; therefore, it seemed useless to study larger groups of animals on diets which indicated only slight protection. Furthermore, partial protection is very difficult to determine because there is variation in incidence of lesions between litters and between lots of flour.

In order to determine whether vitamin $B_1$ was the active factor in rice polish concentrate, another diet (269) which

TABLE 2

*Average body weight and incidence of gastric lesions*

| DIET | AVERAGE WEIGHT | | | | NUMBER OF ANIMALS | | | |
|---|---|---|---|---|---|---|---|---|
| | Experimental animals | | Controls | | Experimental animals | | Controls | |
| | Start | Final | Start | Final | Total | With lesions | Total | With lesions |
| | *gm.* | *gm.* | *gm.* | *gm.* | | | | |
| 151 | .. | .. | 69 | 65 | .. | .. | 155 | 132 |
| 152 | 57 | 63 | 57 | 72 | 6 | 4 | 6 | 3 |
| 154 | 70 | 77 | 71 | 84 | 13 | 5 | 9 | 5 |
| 175 | 72 | 93 | 69 | 67 | 5 | 4 | 4 | 4 |
| 177 | 63 | 73 | 63 | 56 | 4 | 2 | 6 | 6 |
| 178 | 65 | 71 | 63 | 56 | 4 | 4 | 6 | 6 |
| 190 | 58 | 59 | 55 | 51 | 6 | 6 | 3 | 2 |
| 191 | 68 | 57 | 62 | 43 | 5 | 3 | 3 | 3 |
| 199 | 74 | 74 | 76 | 61 | 4 | 2 | 3 | 2 |
| 229 | 67 | 74 | 67 | 57 | 25 | 1 | 22 | 22 |
| 236 | 65 | 55 | 68 | 65 | 6 | 3 | 3 | 3 |
| 237 | 85 | 80 | 85 | 79 | 6 | 6 | 3 | 3 |
| 238 | 70 | 68 | 66 | 53 | 6 | 3 | 3 | 3 |
| 239 | 68 | 68 | 74 | 62 | 5 | 2 | 3 | 2 |
| 240 | 76 | 70 | 76 | 66 | 5 | 4 | 3 | 2 |
| 251 | 70 | 77 | 70 | 60 | 19 | 5 | 15 | 15 |
| 269 | 87 | 82 | 87 | 82 | 10 | 9 | 10 | 5 |

contained 2 mg. per cent of thiamine in place of the 1% rice polish concentrate in diet 229 was studied. Nine of the ten rats fed this diet had lesions. Therefore, the essential supplements to prevent the lesions are lactoflavine, cystine, nicotinic acid, and at least one factor other than vitamin $B_1$, contained in rice polishings.

The effect of the supplements on food intake and the resultant effect on growth bears no relation to production of

the lesions. Rats fed diet 229 grew better than their controls but no better than those fed diet 154, 175 or 178, none of which prevented the lesions.

## DISCUSSION

These experiments along with previous studies (Sharpless, '37) show that the lesions in the forestomach are prevented by a non-specific factor. In a low protein diet cystine may be the preventive factor. In a diet of white flour, cystine plays a minor role and all of at least three members of the vitamin B complex are necessary to prevent the lesions. This non-specific property of the protective substance clarifies some of the confusion that has arisen from studies in which a specific factor was thought to be the protective substance.

The prevention of lesions in the forestomach by a non-specific factor raises two questions which should be discussed in the light of available evidence. First, why do the changes occur in the squamous epithelium of the forestomach and not in other areas of squamous epithelium? Second, is there any evidence that the protective factors have in general any metabolic interrelationship or in particular, any relation to the metabolism of epithelial tissue?

Although the epithelium of the bladder, vagina and pelvis of the kidney is also of the squamous type, there is no sign of lesions in any of these tissues. If the metabolism of squamous epithelium in different parts of the body is the same, the assumption must be made that local conditions account in part for the lesions in the forestomach. Hoelzel and DaCosta have advanced the theory that the primary cause of the lesions is the irritating action of gastric juice. Other studies of our own in which pepsin was fed with and without hydrochloric acid in the drinking water, showed that increase in acidity and pepsin could not completely explain the extreme hyperplasia that occurred with some of the diets. Therefore, regardless of conditions that may be produced in the stomach by the diets studied, it appears that the epithelium has been changed so that less stimulus is required to produce

proliferation. In other words, the threshold of stimulation to proliferation of the epithelial tissue has been lowered.

Evidence that the protective factors have a metabolic interrelationship and that they affect the sensitivity of the cell to proliferative stimuli is fragmentary but certain reports indicate that such relationships may exist. Hueper ('34) has shown that there are many instances in which epithelial tissue is more sensitive to proliferative stimuli when its sulphydryl content is reduced. A decrease in glutathione (and therefore a decrease in sulphydryl) content of various tissues of the rat results from feeding a diet deficient in cystine (Marenzi and Braier, '34) or a diet deficient in vitamin $B_2$ (Itter, Orent and McCollum, '35). Vitamin $B_2$ is now known to consist of at least three fractions, nicotinic acid, lactoflavine, and vitamin $B_6$. These are three of the protective factors; although vitamin $B_6$ was not isolated, it is the other vitamin that is known to be present in abundance in rice polishings. The fact that nicotinic acid cures pellagra with its associated low cystine content of finger nails suggests a relationship between nicotinic acid and sulfur metabolism of epithelial tissue. Recent studies of biological oxidation reduction systems show that the system containing nicotinic acid and the one containing lactoflavine may be concerned with two different phases of metabolism of the same material. Vitamin $B_6$, since it is the rat antidermatitis factor, is known to be concerned with the sensitivity of epithelial cells to proliferative stimuli. This evidence suggests that the non-specific factors prevent the lesions by lowering the sensitivity of the cell to proliferative stimuli and that some of the factors may do this by increasing the sulphydryl content of the cell.

## SUMMARY

Hyperplasia of the squamous epithelium in the forestomach of rats fed a diet of white flour is prevented by adding lactoflavine, nicotinic acid, cystine, and rice polish concentrate to the diet. With a diet of white flour, protein or starvation are not important factors in production of the lesions. The

relation of local irritation to formation of the lesions and the relation of the non-specific protective factors to sensitivity of epithelial cells to proliferative stimuli is discussed.

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# THE STATE OF VITAMIN A IN THE LIVER OF THE RAT AFTER FEEDING VARIOUS FORMS OF THE VITAMIN

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TWO FIGURES

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## INTRODUCTION

In the fish liver, vitamin A occurs as an ester. This was demonstrated for cod in 1928 by Bacharach and Smith who employed aqueous methyl alcohol and petroleum ether to separate the two forms. Employing this same method, Reti, in 1935, showed that the vitamin is present in the ester form in the livers of fish, chickens, and various mammals. In 1936, Hickman, employing distillation and the technique of the elimination curve, showed that in both cod and halibut liver oils, the vitamin A has been largely esterified. Since then, analysis by distillation has been extended to a sufficient number of different species of fish to warrant the generalized statement that vitamin A occurs in all fish-liver oils in the ester form, sometimes accompanied by a trace of the free vitamin. The latter is believed to arise after the death of the fish and to be generated by hydrolysis due to rancidity, the alkali used in refining, or other causes.

It seemed of interest to extend the investigation to mammals in order to obtain some information as to the state of the vitamin stored in the liver and the effect of feeding the different kinds of vitamins. Presumably, the mammal is dependent upon the diet for its vitamin A. Vitamin A can occur in the

diet in three well-defined forms, namely, provitamins, including beta carotene and the other carotenoids, the various esters of vitamin A, and lastly free vitamin A itself. There is thus a clear-cut problem. Does each kind of vitamin in the diet produce a separate selection of vitamin bodies in the liver or do all varieties become stored as the same selection of esters?

It was felt that the analytical technique of molecular distillation was excellently suited for providing an answer to these questions. Since each provitamin or vitamin compound distills at a characteristic temperature identified by the temperature of the elimination maximum, it is possible, by inspecting a distillation map of vitamin A recovered from the liver, to tell at a glance approximately what selection of esters is present and how these differ from the materials administered. For the exploratory work described in this paper, rats were used and the vitamin preparations were administered in massive doses over a short period. It is realized that this procedure is not ideal, and it is planned to extend the study with doses nearer the physiological level. Shortly after feeding, the rats were killed, the livers were removed, and the oil extracted and distilled. Vitamin A was fed in six different forms, as enumerated in the experimental part.

## EXPERIMENTAL PART

Thirteen litters of young white rats from the Sprague-Dawley strain were reared on the Sherman B diet (Sherman and Campbell, '24), the vitamin A in this diet being obtained from whole milk. This ration is sufficient for the early growth, but rats reared on this diet do not store vitamin A in their livers in excessive amounts (McCoord and Luce-Clausen, '34). For our purposes, therefore, we can regard this diet as borderline from the standpoint of liver storage. It was chosen for this reason.

At the age of 53 days, the rats were sampled from each litter and distributed among seven groups to insure approximately equal weight distribution (Luce-Clausen, '29). The vitamin oils were administered by dropper until 20,000 units

of vitamin A had been fed in the various forms during 48 hours. The volume fed varied in each sample group as follows:

| | |
|---|---|
| U.S.P. reference oil [1] | 329 drops |
| Vitamin A caproate | 23 drops |
| Natural ester concentrate [2] | 23 drops |
| Vitamin A stearate | 22 drops |
| Vitamin A alcohol | 20 drops |
| Beta carotene | 212 drops |

The animals were then killed, the livers removed, and those from each group were pooled and weighed. They were then frozen and kept at — 55°C. until used. On examination, each group was thawed and ground up with anhydrous $Na_2SO_4$ and then extracted with ether. Four hundred cubic centimeters of ether were used for the first extraction of each batch, 200 cc. being used for successive extractions. Eight extractions were sufficient to recover substantially all of the vitamin A. The ether solutions were filtered, and the ether removed. With the earlier groups, constant yield oil and residue [3] were added before all of the ether was removed, to minimize destruction of the vitamin. This precaution was found to be unnecessary and so the later samples were taken to dryness, weighed and analyzed before addition of the oil. It was found necessary at some time during the procedure to treat the fatty extract with 50 cc. of n/10 alkali to remove certain nitrogenous constituents, which, if left in the oil, caused spluttering on the still column and interfered with the distillation. The easiest method of treatment was to add the alkali directly to the extract before addition of diluent oil. This material was then shaken out with ether, the ether solution washed with distilled water and dried with anhydrous $Na_2SO_4$ and the ether removed. If the material was alkali-treated after addition of the diluent oil, difficulties with emulsion formation were encountered which made the separation extremely tedious, ac-

[1] In this group the actual dose was 5.8% short due to the refusal of the rats to take the full dose.

[2] Distilled without modification from a rich fish-liver oil.

[3] A high-boiling fraction, free from vitamin A, obtained from the complete distillation of a fish oil.

companied by a certain amount of mechanical loss of oil solution.

After dilution of the extract with constant yield oil and residue, the oils were distilled, using the technique employed in this laboratory (Hickman, '37; Embree, '37). Total vitamin content of each fraction was plotted against the temperature at which the fraction distilled. Curves are given in figure 1. Vitamin A stearate and caproate and distilled ester concentrate furnished similar curves which overlapped in many cases.

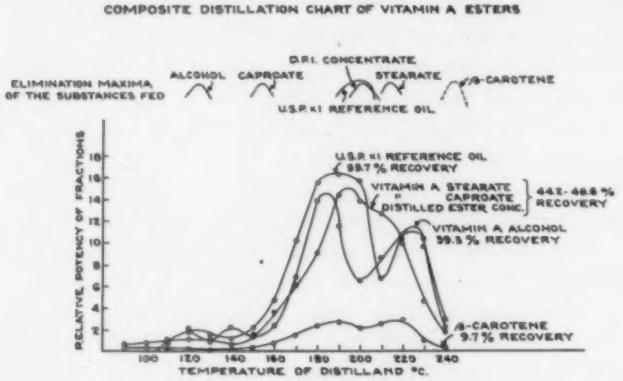
COMPOSITE DISTILLATION CHART OF VITAMIN A ESTERS
D.R.I. CONCENTRATE
ELIMINATION MAXIMA OF THE SUBSTANCES FED
ALCOHOL
CAPROATE
STEARATE
β-CAROTENE
U.S.P. XI REFERENCE OIL
U.S.P. XI REFERENCE OIL 98.7 % RECOVERY
VITAMIN A STEARATE CAPROATE DISTILLED ESTER CONC. 44.2-48.8 % RECOVERY
VITAMIN A ALCOHOL 39.3 % RECOVERY
β-CAROTENE 9.7 % RECOVERY
RELATIVE POTENCY OF FRACTIONS
TEMPERATURE OF DISTILLAND °C.

Figure 1

To present a less confusing picture, they have been plotted as one curve.

The vitamin was assayed by spectrophotometer and by the Evelyn colorimeter. Unfortunately many fractions were too low to display a good absorption spectrum in the ultra-violet, and so the $SbCl_3$ blue color was used in these cases. Where potencies were sufficiently high for spectrophotometric analysis in the ultra-violet, good agreement was obtained with the two methods. The results are summarized in table 1.

## DISCUSSION

The positions of the elimination maxima of the five forms of the vitamin originally fed are indicated at the top of figure

1 for comparison purposes. Since beta carotene was measured as vitamin A, the elimination maximum of the carotene as such is of secondary importance. Only traces of unchanged carotene were recovered from the livers. A small but definite quantity of free vitamin A alcohol is present in all the groups and shows as low maxima at about 120°C. on the curves. As there was not sufficient vitamin present in the control group

TABLE 1

*The recovery of vitamin A in the liver of the rat after feeding various forms of the vitamin*

| SUBSTANCE FED | WEIGHT OF RATS[1] | | WEIGHT OF LIVERS | | WEIGHT OF EXTRACT | TOTAL VITAMIN A FED | TOTAL VITAMIN A[3] RECOVERED | RECOVERY |
|---|---|---|---|---|---|---|---|---|
| | Total | Av. wt. per rat | Total | Av. wt. per rat | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *I.U.* | *I.U.* | % |
| U.S.P. XI reference oil | 1962 | 131 | 94 | 6.7[2] | 3.8 | 273,000 | 152,000 | 55.7 |
| Vitamin A caproate | 1971 | 131 | 87 | 5.8 | .. | 292,000 | 136,000 | 46.6 |
| Distilled ester concentrate | 2001 | 133 | 94 | 6.3 | 3.3 | 288,000 | 127,400 | 44.2 |
| Vitamin A stearate | 2030 | 135 | 88 | 5.9 | .. | 300,000 | 132,800 | 44.3 |
| Vitamin A alcohol | 1996 | 133 | 84 | 5.6 | 3.45 | 300,000 | 118,000 | 39.3 |
| Beta carotene | 2045 | 136 | 92 | 6.1 | .. | 287,000 | 27,700 | 9.7 |
| Controls | 1323 | 132 | 58 | 5.8 | 1.8 | | 4,500 | |

[1] There were fifteen rats in each group except for the controls which had ten rats.

[2] One rat in this group died so the figures for all but weight of rats are based on fourteen rather than fifteen rats.

[3] Due to the potencies involved, the limits of error on the determination are ±15%.

for a distillation, the alcohol: ester ratio was determined by partitioning the lipid between aqueous methyl alcohol (83%) and petroleum ether (Bacharach and Smith, '28; Reti, '35). Approximately 90% of the vitamin was found to be esterified, with 10% in the free form. The small amount of free vitamin always recovered from these rat livers may or may not be significant. Its presence may prove to be as accidental as it

has in the case of the fish-liver oils, but this is considered to be unlikely since it occurs in larger proportions. It seems probable that the A-alcohol serves some physiological purpose not met by the esters or the provitamins. This phase is a subject for further investigation.

The percentage recovery presents a suggestive picture. Since 90–95% of the absorbed vitamin appears in the liver, the vitamin A in this organ may be taken as a measure of vitamin A absorption (McCoord and Luce-Clausen, '34; Moore, '31; and Baumann et al., '34). More accurate data are necessary to prove the exact order of percentage utilization. However, a trend is in evidence with the greatest recovery exhibited from U.S.P. XI reference oil, followed by the separated esters, then the A-alcohol, with beta carotene running a very poor last. The poor utilization of the carotene was visible, large amounts of unchanged provitamin showing in the faeces. Upon autopsy, the entire gastro-intestinal tract appeared bright orange from the pigment. Admittedly large amounts of fat were fed with the carotene, but even larger amounts were fed in the form of U.S.P. XI reference oil which appeared to be the most readily utilized of all. While at small dosage levels, vitamin A and carotene are apparently completely absorbed and utilized, as the dosage is increased, the absorption of carotene and vitamin A fall off, that of carotene falling much lower than that for vitamin A. Davies and Moore ('34), feeding very high levels of vitamin A and carotene, found 15% of the vitamin A absorbed and but 1% of the carotene. Further work on the recovery of these substances in the liver after ingestion at much lower levels will doubtless show whether or not the substances normally fall in the order listed.

A further point of interest is that there is a dip in the ester portion of the elimination curve of the vitamin A recovered from the rat livers, whereas there is no dip in similar curves from the distillation of fish-liver oils. The dip is greatest in the case of cod liver oil, the A-alcohol, and beta carotene; and least in the case of the other esters which give inflexions instead of minima. These curves should be compared with that

for lingcod-liver oil, which is shown in figure 2. There is no minimum here but a trace of inflexion at 200°C., and the curve follows through a maximum at about 220° and falls off. The curve is too broad to denote a simple substance, but evidently represents a whole series of esters, as indeed one would expect to find upon examination of the fatty acids of the glycerides themselves. In contrast to this, the curves from the rat liver oil suggest that some of the fatty acids of intermediate molecular weight are missing, or present only in small quantity. In other words, the liver selects certain specific acids with which to esterify the vitamin to the partial or complete

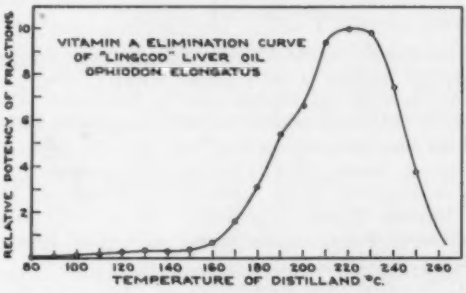


Figure 2

exclusion of others. This is purely a tentative suggestion and may be modified by later findings.

## SUMMARY

1. Vitamin A, fed to rats in the following forms—

U.S.P. reference oil
Vitamin A caproate
Distilled ester concentrate
Vitamin A stearate
Vitamin A alcohol
Beta carotene

was recovered in the livers as the esters, the percentage recovery being in the order of the substances listed. Fifty-five and six-tenths per cent of the vitamin fed as U.S.P. reference

oil was recovered, as against 39.3% recovery from that fed as alcohol and 9.7% recovery from beta carotene.

2. A small amount of the vitamin alcohol was always present, the significance of which is at present not known.

3. The type of ester in each case was similar, which suggests a selective utilization of fatty acids by the rat for the purpose of esterifying the vitamin.

ACKNOWLEDGMENT

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# CHOLINE IN THE DIET OF CHICKENS

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The attention of nutritionists for many years has been directed to the identification of nutrients necessary for growth and reproduction of chickens. Much progress has been made on both the qualitative and quantitative demands but the problem is still one of major interest. Very early in these investigations it was found that simplified diets adequate for rats were inadequate for the satisfactory nutrition of the chick.

In the study of simplified diets Hogan, Boucher and Kempster ('35) described rations which apparently contained all nutrients required by the chick. One of the most promising of these consisted of casein, cornstarch, cellophane, tikitiki, lard, salt mixture, wheat germ oil, acid hydrolized yeast, liver extract, $CaCO_3$; and in addition each chick received daily 0.2 mg. crystalline carotene, and 240 International Units of irradiated ergosterol. Four successive generations were reared on simplified rations. The authors state that the chicks grew rapidly. The males were normal in appearance throughout the period of observation, but their fertility was low; the females were normal until they obtained maturity, but after periods of intensive laying the mortality was high. Their egg records, however, compared favorably with those obtained under normal conditions. From this work it was concluded that if, as indicated by the work of others, the low fertility of the males and the high mortality of the females could be explained on the basis of rearing under laboratory conditions, then the simplified diets were complete in all respects.

It will be noted that while the above diet did not contain lipoids as such, they undoubtedly were present in the extracts of liver and yeast. Moreover, these extracts were found by Hogan, Boucher and Kempster ('35) to be necessary adjuncts to the diet. In the preparation of purified diets it is assumed, however, that animals are able to synthesize lecithin and other lipoids containing phosphorus in large amounts. This assumption is based primarily on the work of McCollum, Halpin and Drescher ('12) and on that of Fingerling ('12).

The recent investigations of Best and his collaborators have stimulated interest in lecithin and its compounds. Best, Hershey and Huntsman ('32) showed that rats fed a daily ration containing 2.5 gm. of fairly saturated fats may produce in the livers large amounts of fatty acids, but if lecithin were added to the diet the liver fat was not in excess of that seen in animals fed a normal diet. Best and Huntsman ('32) then fed separate components of lecithin, but of these, only choline consistently inhibited the deposit of fat in the livers. Later, Best, Huntsman, McHenry and Ridout ('35) found that when rats were fed a ration which provided adequate amounts of protein, fats, carbohydrates and vitamins A, $B_1$, $B_2$, $B_4$ and D, large amounts of fat rapidly accumulated in the livers if the diet did not contain appreciable amounts of choline, or substances having similar effect on liver fat. In this paper evidence was presented which indicated that choline was an accessory food factor.

According to Mathews ('23) egg yolks contain 11% of lecithin, so that 3 gm. of lecithin are necessary for an average yolk. Because of the relatively large lecithin requirement for egg production, the question of lecithin synthesis in the hen is one of importance.

## EXPERIMENTAL

The object of this investigation was to determine the effect of lecithin or choline on the nutrition and egg production of chickens when added to the McCollum, Halpin and Drescher milk-rice diet supplemented with the necessary vitamins.

*Experimental chicks.* Chicks from a high producing strain of Rhode Island Reds incubated in the laboratory of the Department of Home Economics were used in this investigation. They were fed a commercial starting mash until they were 1 week old and from then on the starting mash was gradually replaced by the experimental ration. This usually required about 2 weeks. In the experimental work with pullets the birds were fed the growing mash until they were 16 weeks old and the laying mash until placed on the milk-rice ration at 4 to 6 months of age.

*Quarters.* When the birds were placed on experimental rations they were weighed and put into individual wire cages which were enclosed in a larger tightly-screened, insect-proof cage. This cage had a metal roof but the sides were covered with fine meshed copper wire, allowing the birds to be in the sun the greater part of the day.

*Rations.* The chief components of the basal ration were essentially the same as those used by McCollum, Halpin and Drescher ('12) in their study of lecithin synthesis in the hen—skim milk powder (Merrill-Soule) and polished rice. To these items were added the necessary vitamins, granite grit, oyster shell and cellulose. Following the procedure of McCollum and his associates the rice was ground to a powder and then extracted for 30 minutes with boiling alcohol. Two extractions were made, after which the rice was transferred to a press and nearly freed from alcohol. Finally it was spread in a thin layer until the remaining alcohol evaporated. This diet was considered practically lecithin-free. McCollum found that the ether extract of the milk powder contained about 0.6% of ether-soluble matter and was practically free from phosphorus, while the extracted rice was also relatively fat- and lecithin-free. After a number of experiments to determine the vitamin requirement for pullets, the following diet was found to give the best results: skim milk powder (Merrill-Soule), 30%; rice powder 70%. The vitamin supplements given daily were: 2428 U.S.P. units vitamin A, 485 U.S.P. units vitamin

D, 416 Sherman units vitamin $B_1$, 326 Sherman units of $B_2$ complex.

In feeding chicks, one-fourth of the daily vitamin requirement for pullets was used for the first month and after that the number of vitamin units was gradually increased until the chicks were 3 months old. At that time they were receiving the pullet ration. The lecithin and choline supplements will be given with the experimental data.

*Chemical methods.* The chickens were killed by cutting the carotid artery and the livers removed immediately. The procedure and methods of Best, Channon and Ridout ('34) were followed in the preparation of the samples, and the fatty acids prepared as described by Channon and El Saby ('32).

TABLE 1

*Egg production and condition of 5 to 6 months old Rhode Island Red pullets fed from 195 to 260 days on a simplified diet plus lecithin*

| NUMBER OF PULLETS | DIET | NUMBER OF DAYS ON DIET WHEN LAYING BEGAN | NUMBER OF DAYS ON DIET | AVERAGE NUMBER EGGS LAID PER BIRD | REMARKS |
|---|---|---|---|---|---|
| 12 | Basal | 30–70 | 195 | 16 | 3 hens died; others stopped laying. |
| 20 | Basal + 1 gm. lecithin | 20–60 | 260 | 50 | Hens in fair condition; continued laying. |
| 10 | Laying mash | 20–60 | 275 | 77 | In excellent condition; still laying. |

## RESULTS

The results of feeding chicks and pullets lecithin or choline as an adjunct to a basal diet composed of dried skim milk, rice and vitamins, and the effect of choline on the fat content of the livers, are summarized in tables 1, 2, and 3.

Rhode Island Red pullets 5 to 6 months old fed the basal diet produced eggs for a few weeks, but after that laying became intermittent, and finally ceased. The mortality in this group was high, but when birds on the same diet were given 1 gm. of lecithin per day egg production was consistent and mortality decreased.

Rhode Island Red chicks fed the basal diet from the time they were 2 weeks old grew at a normal rate and a few of them came into laying only a little later than the average of the breed raised on the range and fed commercial laying mash.

TABLE 2

*Egg production and condition of Rhode Island Red chicks raised on a simplified diet plus choline*[1]

| NUMBER OF BIRDS | DIET | AVERAGE NUMBER DAYS UNTIL LAYING BEGAN | AVERAGE NUMBER EGGS PER BIRD DURING FIRST 90 DAYS OF LAYING | CONDITION OF BIRDS AT THE END OF 290 DAYS |
|---|---|---|---|---|
| 10 | Basal | 230 | 5 (only 4 birds laying) | More than half did not lay at all. Livers very fat and soft. Nearly all the egg yolks aborted. |
| 12 | Basal + 25 mg. choline | 190 | 10 | In fair condition. Ovaries showed many aborted egg yolks. Livers very soft and fat. Surfaces hemorrhagic. |
| 12 | Basal + 50 mg. choline | 180 | 22 | In good condition. Few egg yolks aborted. Liver in fair condition. |
| 14 | Basal + 75 mg. choline | 178 | 35 | In good condition. Only an occasional yolk aborted. Liver in good condition. |
| 6 | Basal + 25 mg. choline | | (cockerels) | Testes well developed. Sperms alive and numerous. Livers firm and dark. No abdominal fat. |
| 6 | Basal | | (cockerels) | Testes not so well developed, but sperms were fairly numerous. |
| 7 | Laying mash-range chickens | 210 | 30 | Liver in good condition. |
| 8 | Laying mash-commercial flock | 180 | 35 | In good condition. Livers somewhat soft. |

[1] Choline added when chicks were 3 months old.

But if, when the birds were 3 months old, 75 mg. of choline were given daily their records in regard to age at which laying began and the number of eggs laid were comparable to those of battery-raised birds in commercial flocks. However, when smaller amounts of choline were fed, egg production was likewise smaller.

The choline-fed birds were killed when they were 290 days old and the following observations made: All the pullets had a considerable store of abdominal fat. This fat was firm and white and resembled mutton tallow. The gizzards were very small and were entirely covered with fat. The proventriculum

TABLE 3

*Effect of choline on the fatty acid of chicken liver*

| SAMPLE NO. | SEX | CHOLINE SUPPLEMENT | TOTAL FATTY ACID IN LIVER | FATTY ACID IN LIVER | FATTY ACID IN EXTRACT |
|---|---|---|---|---|---|
| | | *mg.* | *gm.* | % | % |
| 1 | female | 75 | 0.70 | 3.13 | 55.6 |
| 2 | female | 50 | 3.13 | 6.67 | 68.4 |
| 3 | female | 25 | 6.30 | 13.00 | 73.5 |
| 4 | female | none | 8.10 | 15.70 | 73.9 |
| 5 | male | 50 | 0.80 | 3.19 | 52.5 |
| 6 | male | none | 2.80 | 8.22 | 78.6 |
| 7 | female | laying mash | 4.56 | 9.71 | 72.5 |

was also covered with fat and was abnormal in that it was not constricted at either end. The oviducts and uteri were well formed and appeared normal but the ovaries of the birds fed diets deficient or low in choline showed many aborted egg yolks. Abortion had evidently taken place when the yolks were about two-thirds developed. On the other hand, the ovaries of the birds fed as much as 50 to 75 mg. of choline showed very few aborted yolks.

While the livers of all the pullets were large and soft and light in color, the ones from the birds getting little or no choline were softer and lighter in color than those from birds receiving 50 to 75 mg. of choline. In the former group the

livers showed hemorrhagic surfaces and appeared very fatty. In addition to the effects of choline already noted, the analytical data show that choline inhibits the accumulation of fatty acids in the livers. It will be noted that the concentration of fatty acids in the livers of the males fed diets with or without choline was considerably lower than that in the livers of pullets fed the same rations.

## DISCUSSION

In the preliminary experiments the adjustment of the vitamin content of the ration presented the greatest difficulty. Whether the ration as now prepared has an optimum vitamin content awaits further feeding trials. At no time during the experiments did recognizable symptoms of vitamin D deficiency appear. This probably was due to the fact that in addition to the vitamin in the ration the birds were in the sun the greater part of the day. On the other hand, loss of appetite, crop stasis, paralysis and other symptoms indicative of a lack of $B_1$ or some part of the $B_2$ complex were common until the daily vitamin intake was 416 Sherman units of $B_1$ and 326 units of $B_2$ complex. Even with this intake, occasionally a bird showed symptoms indicative of a lack of some part of the vitamin B complex. Likewise, symptoms of vitamin A deficiency, as evidenced by eye defects and a condition resembling roup were common until 2428 U. S. P. units of vitamin A were given each bird daily.

It is not postulated, however, that the amounts of the various vitamins necessary for growth and reproduction of these birds are the amounts that represent the average requirement of birds on regular poultry rations.

As to the optimum amount of the choline, the other dietary factor, little is known. The data show that when 75 mg. of choline were administered daily, pullets produced more eggs and there were fewer aborted yolks than when 25 to 50 mg. were given. Whether chicks are able to synthesize any or all the lecithin or choline necessary for optimum growth and reproduction is open to question. Best and his collaborators ('34)

are of the opinion that if the rat can synthesize these constituents, the amount is limited. But McCollum, Halpin and Drescher ('12) present data which they interpret as proving "that the synthesis of phosphatides is readily accomplished in the body of the hen when the ration is free from these substances." However, when McCollum's experiment was repeated in this laboratory it was found that, on a diet composed of only rice meal and dried skim milk, 5 to 6 months old pullets laid for a short time, developed symptoms of multiple vitamin deficiency and died. When younger birds were fed the diet, they quickly showed symptoms of vitamin deficiency and did not lay at all. It was evident that stored vitamins and perhaps other constituents necessary for life and reproduction enabled the older birds to lay for a time; but as soon as the stores were exhausted laying stopped and the birds declined, while the younger birds with less stored nutrients died quickly unless the diet was changed.

The data showing that the concentration of fatty acids in the livers of the pullets is greater than that in the livers of the cockerels is of interest. Okey and Yokela ('35) found also that the fatty acid in the livers of female rats fed egg yolks for 120 days was almost three times that of the males. They point out that the fatty acid concentration in the livers of the males decreased with age while that of the females increased. This variation in the accumulation of fatty acids in the livers of male and female rats and in the livers of cockerels and pullets offers interesting possibilities for speculation and suggests some sex variation in the utilization of choline or lecithin. In a preliminary study of a female sex hormone made in this laboratory it was noted that the hormone injected in oil was readily absorbed, but when the oil alone was injected it was very poorly absorbed. Whether the above phenomenon was due to action of the female sex hormone is suggested for consideration. However, in examining livers from non-laying and laying hens and mature cockerels, all fed a commercial feed, differences in size, color and fat content are striking.

Whether choline is a contributing or causative factor is not known.

## CONCLUSION

1. The addition of choline to the basal diet increased egg production, decreased mortality, inhibited abortion of egg yolks, and decreased the percentage of fatty acids in the livers.

2. The concentration of fatty acids in livers of the males fed diets with or without choline was considerably lower than that in the livers of pullets fed the same rations.

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# QUANTITATIVE REQUIREMENTS OF THE COMPONENTS OF THE VITAMIN B COMPLEX FOR LACTATION AND GROWTH OF NURSING YOUNG OF THE ALBINO RAT[1]

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SIX FIGURES

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The investigations of the dietary requirements for fertility and lactation begun in the University of Wisconsin in 1918 and in this laboratory in 1920 were suspended in 1932 awaiting isolation of the various components of the vitamin B complex. It was realized in 1938, of course, that, although $B_1$ (thiamin), riboflavin, and $B_6$ have been isolated in pure form as yet unidentified members of the B complex remain to be isolated. However, they may be provided by means of extracts relatively free from known substances. Use of these extracts permits one to construct diets with unknown factors reduced to a minimum. Therefore, lactation studies were resumed last year, and attempts were made to rear nursing young of the albino rat on diets containing the purest available components of the vitamin B complex.

All early attempts to determine the vitamin $B_1$ requirements for lactation with diets containing autoclaved products, such as Northwestern yeast, autoclaved beef, and autoclaved liver, resulted in failure in most trials. As much as 15% autoclaved liver and 10% autoclaved beef did not always insure normal

[1] Research paper no. 622, Journal series, University of Arkansas. This is paper XXVI in the series Dietary Requirements for Fertility and Lactation.

rearing of young, even when supplemented with 60 μg. of thiamin daily. It became apparent that considerable destruction of components of the vitamin B complex, other than $B_1$, occurs during autoclaving, and in order to satisfy the requirements of such dietary essentials for lactation, the procedure of using natural foods cooked under pressure had to be abandoned.

When an attempt was made to prepare the synthetic vitamin B complex mixture, vitamin $B_1$[2] and riboflavin[3] were available in pure crystalline form. Vitamin $B_6$ had just been isolated by Lepkovsky ('38) and independently by Keresztesy and Stevens ('38), and by György ('38). György and co-workers had previously reported ('37) that Peter's eluate, free from riboflavin, was a dependable source of vitamin $B_6$, 1 cc. being equivalent to 10 gm. of yeast, and that this eluate contained an additional substance, termed the 'maturation factor.' A deficiency of the 'maturation factor', according to György and associates, produced panmyelophthisis in the rat ('37). Since the procedure employed for the preparation of '$B_6$ and the maturation factor' was the one originally used by Kinnersley and associates ('33) for the production of $B_4$, it was considered that, if $B_4$ is essential for the rat as claimed by Kline and associates ('36), it would be provided by Peter's eluate.

It soon became evident, however, that the '$B_6$ and maturation factor' extracts were not a dependable source of $B_6$ for lactation purposes, and furthermore, they were much too expensive to permit numerous experiments. A continuation of the work was then made possible by generous amounts of $B_6$ crystals furnished by Dr. S. Lepkovsky. Since the chick antidermatitis factor of Lepkovsky and co-workers ('36) had not been established as a dietary essential for the rat, it was not used. The Peter's eluate ($B_6$ and maturation factor) furnished a component other than thiamin, riboflavin, $B_6$, choline and nicotinic acid. This component, however, was found in a much more concentrated form in preparations designed to

[2] Generously supplied by Merck and Company.
[3] Generously supplied by Hoffman-LaRoche Company.

furnish the 'W' factor of Elvehjem, Koehn, and Oleson ('36). Choline was used in the synthetic vitamin mixture, since McHenry ('35), and Best and associates ('36) have reported its presence to influence the growth of rats under certain dietary restrictions. Although nicotinic acid, found to be essential for the prevention and cure of black tongue in dogs (Elvehjem, Madden, Strong and Woolley, '38), and to be the human antipellagric factor (Smith and associates, '37; Spies and co-workers, '38; Bogart, '38) had not yet been shown to be essential for the rat, it was incorporated in the vitamin mixture, in order to be sure that it would not be a limiting factor. The nursing mothers received 6 mg. nicotinic acid daily.

In order positively to satisfy the protein requirements for lactation, the proportion of casein in the ration was increased from 20% (used in earlier work) to 25% supplemented with 0.2 to 0.5% cystine (see rations 6 and 7, table 1). This procedure was suggested by the work of Daggs and Lidfeldt ('38). When it became apparent by the losses of weight of the mother and litter that a dangerous period of depletion of vitamin reserves had been reached,[4] supplements of the various components of the vitamin B complex were given daily separately from the ration. Ten drops a day of cod liver oil[5] supplied vitamins A and D. Choline was furnished in the form of the chloride, 15 mg. being the dose used daily.[6]

Space does not permit the report here of our extensive data. Typical lactation records, representing about one-sixth of the total data, are submitted graphically in figures 1 to 6 inclusive. The composition of rations is given in table 1. Growth records of mothers and litters were taken daily. Food consumption records were also taken daily, but are not reported here in order to conserve space.

[4] The dietary technic employed was that outlined in previous publications (Sure, '28 a, b, c).

[5] Obtained from Mead Johnson and Company.

[6] Obtained from The Eastman Kodak Company.

*Lactation efficiency on stock diet no. 1*

Stock diet no. 1 is a cereal ration containing 1% cod liver oil, 5% commercial casein, 0.5% NaCl and 0.5% $CaCO_3$. It is supplemented 6 days a week with 5 cc. fresh cow's milk per animal until breeding, when the amount is increased to 10 cc., and during lactation, when the young begin eating, to 15 cc. daily. Each animal also received 15 gm. fresh lettuce once weekly. On such a dietary regime about 95% of the young are raised. During the nursing period the mother either main-

TABLE 1

*Composition of rations*

| | *No. 6* | *No. 7* | *No. 8* |
|---|---|---|---|
| Casein[1] | 25.0 | 25.0 | 25.0 |
| Agar-agar | 2.0 | 2.0 | |
| Salts 185[2] | | | 4.0 |
| Salts 351[3] | 4.0 | 4.0 | |
| Cystine | 0.2 | 0.5 | |
| Brewer's yeast | | | 2.0 |
| Butterfat | 10.0 | 10.0 | 10.0 |
| Dextrin | 58.8 | 58.5 | 44.0 |
| Liver (autoclaved 6 hrs. at 15 lbs. pressure) | | | 15.0 |

[1] Extracted several times with hot 95% alcohol and cold 25% alcohol.

[2] McCollum, E. V., and Nina Simmonds 1918 A study of the dietary essential, water-soluble B, in relation to solubility and stability towards reagents. J. Biol. Chem., vol. 33, p. 63.

[3] Hubbell, Rebecca B., Lafayette B. Mendel and Alfred J. Wakeman 1937 A new salt mixture in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

tains her weight or may gain 8 to 10% of the body weight at parturition. The young open their eyes about the fifteenth day and begin eating of the maternal diet between the seventeenth and twentieth day. The litter is considered weaned when each young rat weighs 40 gm. which generally takes place the twenty-second to the twenty-fifth day of lactation (fig. 1). The dotted line curve in the figures submitted indicates the body weight of the nursing mother whereas the heavy line curve denotes the growth of the litter. In determining the optimum amount of any of the components of the vitamin B complex

essential for lactation, or in the consideration of the need of additional vitamin essentials for milk secretion, the lactation efficiency index on stock diet no. 1 was used as a basis for comparison. Appropriate reference curves indicating this standard performance are shown in the respective figures.

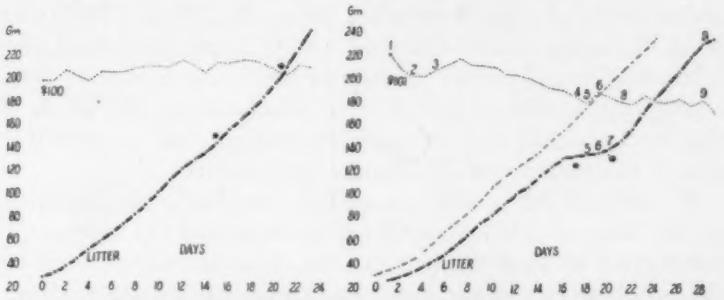


Fig. 1 Lactation record of ♀100 (159 days old) on stock diet no. 1. *, young opened their eyes. #, young eating.

Fig. 2 Lactation record of ♀101 (171 days old). Showing vitamin $B_1$ requirements for lactation and for growth of nursing young. 1, ration no. 6; 2, ration no. 8; 3, ration no. 7, supplemented with 120 μg. riboflavin, 50 μg. vitamin $B_6$, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W factor to mother; 4, 60 μg. $B_1$ to mother; 5, 18 μg. $B_1$ to mother, and 42 μg. $B_1$ to litter; 6, 18 μg. $B_1$ to mother, and 60 μg. $B_1$ to litter; 7, 90 μg. $B_1$ to litter; 8, 30 μg $B_1$ to mother; 9, remove all vitamin supplements from mother and give litter 120 μg $B_1$. *, young opened their eyes. #, young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

### *Thiamin and riboflavin requirements for lactation*

In chronic vitamin $B_1$ deficiency produced on rations containing autoclaved yeast and beef (Sure, '38) 10 μg. of thiamin[7] was always sufficient for the cure of polyneuritis associated with marked convulsions, and was always accompanied by continuous and satisfactory growth. In attempting to determine the thiamin requirement for lactation on rations containing 25% autoclaved yeast and beef or autoclaved liver and beef, as much as 60 μg. $B_1$ did not definitely assure normal rearing of the young. After the completion of numerous ex-

[7] Thiamin and $B_1$ are used interchangeably in this paper.

periments it became evident that 120 μg. of thiamin had to be included in the synthetic mixture of the components of the vitamin B complex to prevent polyneuritis in the young and to insure weaning of the litter at a rate comparable to that secured with our stock diet no. 1 (Sure, '26).

After it became evident that, following depletion of the reserves of the vitamin B complex, 120 μg. $B_1$, 120 μg. riboflavin, 50 μg. $B_6$, 15 mg. choline chloride, 1 cc. $W^8$ factor, and 6 mg. nicotinic acid would permit rearing of the young at a rate very nearly comparable to that secured on our stock diet no. 1, it was then possible to vary quantitatively any of the constituents in this mixture of the vitamin components.

From figure 2 it is clear, judging by the character of growth of the litter of ♀ 101, that 60 μg. $B_1$ furnished the mother or distributed between mother and young on the seventeenth to the twentieth day of lactation, was inadequate for the continuous growth of the nursing young. Desired growth was accomplished only after the litter received 90 μg. $B_1$ and the mother 30 μg. $B_1$. In numerous other experiments litters were reared on a daily dose of 120 μg. $B_1$ given to the mother but in many instances the vitamin had to be distributed between young and mother, allowing about three-fourths of the daily dose to the litter and one-fourth to the mother. This offers corroborative evidence of former experiments (Sure, '28a) of the inefficiency of the lactating mother in transferring vitamin $B_1$ to the milk. Such a phenomenon will become more apparent from an analysis of the other figures. That such inefficiency is not confined exclusively to thiamin will also become evident from an examination of the rest of the data.

Figure 3 shows that growth of the litter of ♀ 102 was tremendously retarded until the daily dose to the mother was increased to 90 μg. riboflavin.

From figure 4 it is evident that, whereas 90 μg. riboflavin failed when given to the mother, a distribution of 30 μg. to the mother and 60 μg. to the litter yields successful results. The

[8] Equivalent to 1 gm. of Wilson's liver extract.

first plateau in the curve of growth of the litter was reached between the twenty-seventh and thirtieth day of lactation because of riboflavin deficiency. The second plateau appeared on the thirty-fifth day when increased allowance of riboflavin to the litter brought no response but a distribution of vitamin $B_1$ between mother and young resulted in increased growth.

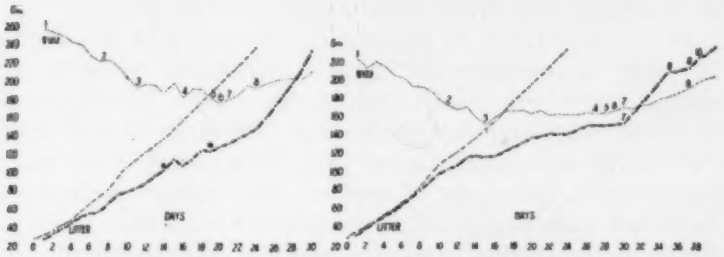

Fig. 3 Lactation record of ♀102 (191 days old). Showing riboflavin requirements for lactation. 1 Ration no. 7. 2 120 μg. $B_1$, 50 μg. vitamin $B_6$, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W factor to mother. 3 10 μg. riboflavin to mother. 4 20 μg. riboflavin to mother. 5 30 μg. riboflavin to mother. 6 40 μg. riboflavin to mother. 7 60 μg. riboflavin to mother. 8 90 μg. riboflavin to mother. * Young opened their eyes. # Young eating. Curve in thin line represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Fig. 4 Lactation record of ♀103 (167 days old). Showing riboflavin requirements for lactation and for growth of nursing young. 1 Ration no. 7. 2 120 μg. $B_1$, 50 μg. vitamin $B_6$, 15 mg. choline, 6 mg. nicotinic acid, and 1 cc. W factor to mother. 3 20 μg. riboflavin to mother. 4 30 μg. riboflavin to mother. 5 60 μg. riboflavin to mother. 6 90 μg. riboflavin to mother. 7 30 μg. riboflavin to mother, and 60 μg. riboflavin to litter. 8 90 μg. riboflavin to litter. 9 30 μg. $B_1$ to mother, and 90 μg. $B_1$ to litter. 10 Remove $B_1$ from mother and give 120 μg. $B_1$ to litter. * Young opened their eyes. # Young eating. ♦ Alopecia in young. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

### *Vitamin $B_6$ requirements for lactation*

From the results of eight lactation experiments it became evident that it is necessary to introduce at least 50 μg. of $B_6$ for lactation, in order to rear young at a rate approximating that obtained on stock diet no. 1 (fig. 1). For continuous growth 10 to 25 μg. $B_6$ were found adequate. To conserve space, however, such data are not presented here.

### *The 'W'[9] factor versus 'B$_6$ and maturation factor' for lactation*

A biological assay of the preparation used to supply the 'W' factor for various components of the vitamin B complex disclosed that it contained small amounts of vitamin $B_6$ for growth but negligible amounts for lactation. It was found to be practically free from thiamin and riboflavin.

In the early trials an attempt to rear young of the albino rat with supplements of thiamin, riboflavin, vitamin $B_6$, choline, and nicotinic acid resulted in complete failure about the tenth to fifteenth day of lactation. The addition of 3 cc. of the '$B_6$ and maturation factor' extract[10] resulted in the rearing of three litters out of twelve. This extract must have furnished some component or components other than thiamin, riboflavin, $B_6$, choline, or nicotinic acid. It was then decided to introduce the 'W' factor of Elvehjem and associates ('36, '37). This resulted in success in every experimental trial. It was found that 1 cc. of the solution containing the 'W' factor was many times more potent than 3 cc. of that which furnished the '$B_6$ and maturation factor.' This is brought out in figure 5 (see eighteenth day). The accelerated growth of the litter after the daily dose of thiamin was increased to 120 μg. is also apparent.

From figure 6 it is apparent that lactation proceeds at a rate comparable to that secured on stock diet no. 1 when the synthetic mixture of the components of the vitamin B complex given the nursing mother contains 120 μg. $B_1$, 120 μg. riboflavin, 50 μg. $B_6$, 15 mg. choline chloride, 6 mg. nicotinic acid, and 1 cc. of a solution containing 'W' factor, equivalent to 1 gm. of liver extract. The litter of ♀105, which was reared in 24 days, would have been weaned sooner if there had not been

[9] The 'W' factor was prepared from liver extracts furnished by Dr. David Klein of the Wilson Laboratories, according to the technic of Frost and Elvehjem ('37).

[10] Furnished by the S.M.A. Corporation. The 3 cc. of extract are equivalent to 30 gm. yeast, prepared according to the method of Kinnersley and associates ('33) and referred to in the literature as Peter's eluate.

a depletion period of 4 days on the maternal diet in this experiment. One change was made in this instance, namely, on the seventeenth day of lactation when the young were growing at a subnormal rate, the 120 μg. dose of $B_1$ was distributed between mother and young, one-fourth to the former and three-fourths to the latter. Following this change the litter exhibited accelerated growth.

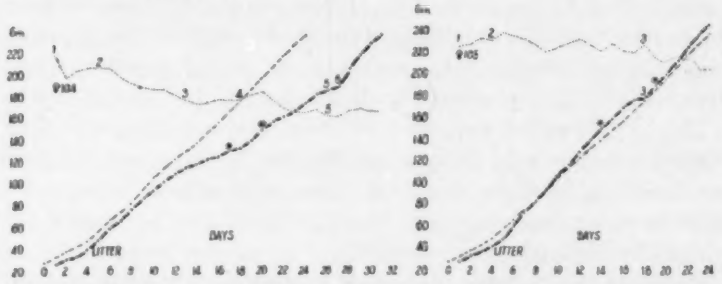


Fig. 5 Lactation record of ♀104 (120 days old). Showing the potency of the W factor versus the $B_6$ and maturation factor extract for lactation; also the inefficiency of this mother in secreting $B_1$ in the milk. 1 Ration no. 7. 2 120 μg. $B_1$, 120 μg. riboflavin, 50 μg. crystalline $B_6$, 15 mg. choline, 6 mg. nicotinic acid to mother. 3 3 cc. $B_6$ and maturation factor extract to mother. 4 Replace the 3 cc. $B_6$ and maturation factor extract with 1 cc. W factor extract. 5 30 μg. $B_1$ to mother, and 90 μg. $B_1$ to the litter. 6 Increase to 120 μg. $B_1$ to the litter. * Young opened their eyes. # Young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Fig. 6 Lactation record of ♀105 (256 days old). Showing that lactation proceeds at a rate comparable with that obtained on stock diet no. 1 (fig. 1) on a synthetic mixture of the vitamin B complex when adequate amounts of the various components are furnished as outlined below. 1 Ration no. 6. 2 Ration no. 7, supplemented with: 120 μg. $B_1$, 120 μg. riboflavin, 50 μg. $B_6$, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W to mother. 3 30 μg. $B_1$ to mother and 90 μg. $B_1$ to litter. * Young opened their eyes. # Young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

## DISCUSSION

The results of the studies reported in this paper deserve some comment. As far as the author is aware, these are the first lactation investigations carried out with the use of synthetic diets free from autoclaved natural foods. Four vitamins, which are recognized as dietary essentials for the rat,

dog and man, namely, thiamin, riboflavin, $B_6$, and nicotinic acid, have been used in pure form. In addition, a fifth product, choline chloride, has been included in the synthetic mixture of the components of the vitamin B complex, which must now be recognized as essential for the rat (Sure, '40).

Since the completion of the work reported here, six litters have been successfully reared without nicotinic acid administered either to the mother or to the young. Neither could it be demonstrated in this laboratory that nicotinic acid is essential for the weaned rat subsisting on highly purified diets. Our results are in accord with the recent findings of Birch ('39). Frost and Elvehjem ('39) however, reported that they found nicotinic acid to be essential for the rat when they introduced 12% white corn in their rations and interpreted this to mean that the corn furnishes a factor necessary for the utilization of nicotinic acid.

Several litters have also been reared on our synthetic ration containing 25% casein without cystine additions. This high per cent of casein probably furnishes enough methionine to make it a complete protein for lactation.

If there exist needed components of the vitamin B complex other than thiamin, riboflavin, $B_6$, and choline, these unidentified substances must have been furnished by the solution containing 'W' factor, which was prepared from liver extracts (Wilson). For example, the spectacled appearance observed by Oleson and associates ('39), the panmyelophthisis reported by György and co-workers ('37) and the $B_4$ deficiency reported by Kline and associates ('36), have not been observed in the nursing young in this investigation. Perhaps the symptomatology nearest to that of $B_4$ deficiency observed here was the paralysis in nursing young encountered in choline deficiency. In this connection it may be mentioned that McHenry ('35) considered the symptoms exhibited by his choline deficient rats to resemble those of $B_4$ deficiency as described by Reader ('29, '30 a, b).

The much greater requirement of thiamin, riboflavin, and $B_6$ for lactation may perhaps be best explained as due to the

utilization of greater amounts of food ingested during this period. This explanation, however, is not entirely satisfactory, because, although the food intake during lactation is doubled or trebled, the thiamin and riboflavin requirements for rearing of young are at least five to six times that required for growth on highly purified diets free from autoclaved foods and ten to twenty times that necessary for the cure of such avitaminotic symptoms as polyneuritis and cataracts. Enough vitamin must be supplied not for one individual but for the mother and the rapid development of six individuals, since, according to the method, each lactating mother is allowed six young to rear.

Another puzzling problem that awaits solution is the difficulty many mother rats have in transferring to the milk the pure thiamin, riboflavin, $B_6$, and choline chloride, a phenomenon not observed when satisfactory diets are composed of natural foods. It may be of interest to relate here an experience noted in several lactation experiments. After the publication of the recent report of Perla ('39) that manganous chloride exerts a protective action against injurious effects of massive doses of vitamin $B_1$ on lactation, this salt was given to mother rats which were inefficient in transferring thiamin to the milk. In three instances a daily dose of 2 mg. manganous chloride permitted the mothers to rear their litters without the necessity of distribution of the vitamin between mother and young.

## SUMMARY

1. A study has been made of the amounts of various components of the vitamin B complex needed for successful lactation.

2. Following depletion of vitamin reserves, it has been determined that rearing of the young of the albino rat can proceed at a rate comparable with that secured on an optimum stock diet when the following supplements are furnished daily to the mother: 120 μg. $B_1$ (thiamin), 120 μg. riboflavin, 50 μg. $B_6$, 15 mg. choline chloride, 1 cc. of a solution of 'W' factor

equivalent to 1 gm. of liver extract, and 6 mg. nicotinic acid. It was not determined whether the nicotinic acid is really essential for the lactating rat.

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# THE ESSENTIAL NATURE OF CHOLINE FOR LACTATION AND GROWTH OF THE ALBINO RAT[1]

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THREE FIGURES

(Received for publication August 28, 1939)

The lipotropic action of choline has been demonstrated by Best and co-workers ('34, '35 and '36) and confirmed by Perlman and Chaikoff ('39). McHenry ('35) and Best and associates ('36) have also produced evidence that choline influences the growth of rats. The latter workers emphasized that, in order to demonstrate effects of choline deficiency the diet must contain a high proportion of fat (40%) and a low concentration of protein, if casein is the source of nitrogen in the ration because of the lipotropic effect of that protein. The average period of experimental study was about 21 days. The results of the work of these investigators suggested the introduction of choline into the mixture intended to supply the components of the vitamin B complex. It was decided to test the effect of the daily administration of 15 mg. choline chloride during lactation. This was found satisfactory in most instances. In several cases rearing of the young to weaning was accomplished only after this dose was divided between mother and young. The diet had the following composition: casein (extracted several times with hot 95% alcohol) 25%; agar-agar 2.0; salt mixture 4 [2]; cystine 0.5; butterfat 10; dextrin 58.5%.

[1] Research paper no. 623, journal series, University of Arkansas. This is paper XXVII in the series of Dietary Requirements for Fertility and Lactation.

[2] Hubbell, Rebecca B., Lafayette B. Mendel and Alfred J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

In addition each mother received daily 120 μg. thamin, 120 μg. riboflavin, 6 mg. nicotinic acid, and 1 cc. of a solution containing the 'W' factor equivalent to 1 gm. liver extract (Wilson).

In figures 1 and 2 are presented lactation records typical of those yielded by six mothers. They show that choline is a dietary essential for the growth of nursing young of the albino rat. Figure 1 is a lactation record showing depletion of choline on the thirteenth day and the response obtained in the growth of the litter following an administration of 15 mg. choline chloride daily. Before the choline was given the young developed paralysis similar in symptomatology to that described by McHenry in weaned rats ('35) and Reader ('29

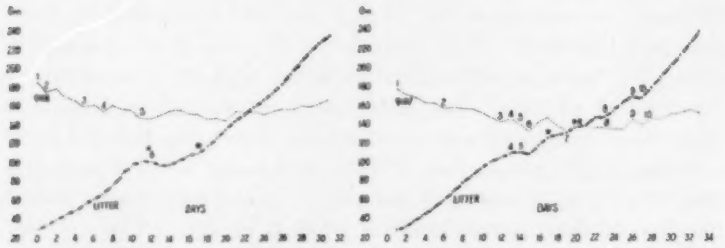

Fig. 1 Lactation record of ♀108 (85 days old). Showing choline to be an essential dietary factor for growth of nursing young of the albino rat. 1, stock diet no. 1; 2, ration no. 22,[1] 3, ration no. 7; 4, 120 μg. $B_1$, 120 μg. riboflavin, 25 μg. $B_6$, and 0.5 cc. W factor to mother; 5, 50 μg. $B_6$ and 1 cc. W factor to mother; 6, 15 mg. choline to litter. *, young opened their eyes. #, young eating.

Fig. 2 Lactation record of ♀107 (155 days old). Showing that choline is an essential component of the vitamin B complex for growth of nursing young of the albino rat; also that this mother was inefficient in secreting this dietary essential in the milk. 1, ration no. 7; 2, 120 μg. $B_1$, 120 μg. riboflavin, 50 μg. $B_6$, 15 mg. choline to mother; 3, 6 mg. nicotinic acid to mother; 4, 30 μg. $B_1$ to mother and 90 μg. $B_1$ to litter; 5, 30 μg. riboflavin to mother, and 90 μg. riboflavin to litter; 6, 3 cc. $B_6$ and maturation factor extract to mother; 7, replace the 3 cc. $B_6$ and maturation factor with 1 cc. W factor to mother; 8, 20 μg. crystalline $B_6$ to mother and 30 μg. crystalline $B_6$ to litter; 9, 10 μg. $B_6$ to mother and 40 μg. $B_6$ to litter; 10, 5 mg. choline to mother, and 10 mg. choline to litter. *, young opened their eyes. **, young eating.

[1] Composition of ration no. 22 is as follows: Casein (extracted with hot alcohol) 25; agar-agar, 2; salts 351, 4; Crisco, 10; dextrin, 59.0.

and '30). The paralysis disappeared the day following the administration of choline. Apparently the mother did not need choline, because she maintained her body weight from the time the choline therapy to the litter was begun.

From figure 2 it is evident that, although the litter of ♀107 began partaking of the maternal diet the twentieth day of lactation, satisfactory growth of the young was not secured until the daily dose of 15 mg. choline chloride was distributed between mother and young on the twenty-seventh day of lactation, allowing 5 mg. to the former and 10 mg. to the latter.

Although the choline deficiency in the nursing young of the rat in this study was produced on a ration containing 10% fat, it should be taken into consideration that the milk of the rat contains 32% fat (Donaldson, '24). Therefore, the nursing young were on essentially a high-fat diet.

In a number of cases choline chloride was withheld from the vitamin B complex mixture, and this resulted in the death of the litters about the fifteenth to the eighteenth day of lactation. The avitaminotic state in the nursing young of the rat is characterized chiefly by paralytic symptoms indistinguishable from $B_1$ polyneuritis in litters of the same age, but without associated running screaming fits (Sure, '28).

Following the accumulation of evidence that choline is an indispensable dietary factor for the growth of nursing young, attempts were made to determine whether choline is essential for growth of the weaned rat, employing our own dietary technique. McHenry's work ('35) may be criticized on the ground that vitamin $B_1$ is the only constituent he introduced to satisfy the requirements of the vitamin B complex. Furthermore, neither McHenry nor Best and associates ('36) established that their synthetic rations contained sufficient riboflavin or $B_1$. In the construction of our purified diets it was felt desirable to avoid the introduction of a natural food such as egg-white. The early experiments, involving 116 animals fed rations consisting of 20% purified casein and 10% fat, yielded inconsistent results. After some correspondence with Doctor Best, it was decided to use a high-fat low-protein

diet, and a ration was constructed, on which choline deficiency has been produced in twenty-four groups of animals. The composition of the diet was as follows: fibrin (extracted with hot alcohol) 9%; agar-agar 2; salt mixture 4; Crisco 30; and

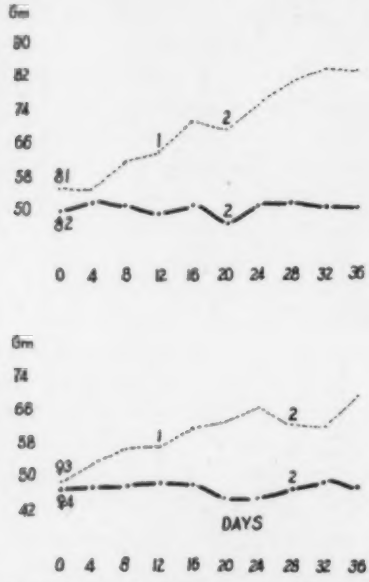


Fig. 3 Showing the influence of choline on growth of weaned rats on a diet containing 9% fibrin and 30% fat (Crisco). Straight line curves represent the growth of the animals on a diet deficient in choline. Dotted line curves represent the growth of litter mates of the same sex which were restricted to the same amount of food consumed by the choline-deficient rats, but which in addition received 6 to 15 mg. choline chloride daily.

[1] Increased choline chloride from 6 to 15 mg. daily.

[2] One-tenth cubic centimeter W factor daily (equivalent to 0.1 gm. liver extracts).

sucrose (extracted with hot alcohol) 55.0%. The majority of the experiments were conducted by the paired feeding method, the control litter mate animals of the same sex being restricted daily to the amount of food consumed by those receiving the choline-deficient diet. Each animal received daily separately

from the ration 20 μg. thiamin, 20 μg. riboflavin, and 10 μg. $B_6$. The control received in addition 6 to 15 mg. daily of choline chloride. Two typical cases are presented in figure 3. Several of the choline deficient animals which died were found at autopsy to have hemorrhages in the cortex of the kidneys which is in agreement with the recent findings of Griffith and Wade ('39). After about 4 to 6 weeks of growth, the control as well as the choline-deficient animals fail to gain because of the deficiency of other components of the vitamin B complex. The supplementation with 0.1 cc. to 0.2 cc. of the solution containing the 'W' factor brings a response, but often the choline-deficient animal also resumes growth. This probably means that the solution used to supply factor 'W' also furnished the lipotropic factor, choline chloride or some other substance. We are observing at this time that the change from purified sucrose to dextrin exerts a stimulating effect on growth, as recently reported by Oleson and associates ('39).

## SUMMARY

Choline has been found to be an indispensable component of the vitamin B complex for growth and lactation of the albino rat.

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# THE INFLUENCE OF THE NITROGEN CONTENT OF THE DIET ON THE CALORIE BALANCES OF PRE-SCHOOL CHILDREN [1]

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ONE FIGURE

(Received for publication September 2, 1939)

Although there have been many studies showing the calorie needs of pre-school children, there have been few balance studies which determined both the actual number of calories obtained from food and those lost in the excreta. Such studies give accurate values for available calories and show the influence of various factors upon availability.

In connection with their studies on the metabolism of undernourished children, Wang and her associates ('28 a, '28 b and '29) determined 3-day calorie balances. They calculated the intake values (Rose, '25), analyzed the urine and feces by means of the Bomb calorimeter and determined the available calories by difference. Although values for calorie intake calculated by means of physiological values from which excretory losses had been subtracted may vary considerably from analyzed figures (Donelson and her associates, '31; Bray, Hawks and Dye, '34), the results of Wang and her associates indicate several interesting trends. All of the

[1] Published from Michigan State College Agricultural Experiment Station as paper no. 381, new series.

[2] Some of the data reported in this paper were presented by Jeanne M. Voorhees in partial fulfillment of the requirements for the degree of master of science, Michigan State College.

[3] Some of these data were presented before the American Home Economics Association, Chicago, Illinois, June, 1935.

children they studied, both those normal in weight and undernourished, absorbed and retained approximately the same percentage of the calorie intake, indicating that the calorie loss for 24 hours, both in urine and feces, increased with intake. This constant percentage utilization was the same whether the children did or did not gain in weight. At the same time the results suggest that there may be a relationship between the protein and calorie contents of the diet, because, when the protein content was increased the results showed an increase in eliminated calories, both in urine and feces. In view of these findings, it would seem that balance studies, conducted over long periods of time and which gave actual values, might produce further information on the availability of calories and the effect of diet on calorie balance.

The purposes of the present study were to determine whether small period-to-period variations in intake calories influence their availability and to ascertain the effect on calorie balance of increasing the protein content of the diet. Before these purposes could be accomplished, however, it was necessary to determine the length of time required for a preliminary period, especially following a change in the protein content of the diet and also to learn whether slight differences in the composition of diets would be sufficient to cause variations in results.

## EXPERIMENTAL PROCEDURE

The data on calorie balances given in this paper, as well as those for nitrogen utilization discussed in a previous paper (Hawks, Bray and Dye, '38) were obtained during two long-continued balance studies on five pre-school children. For the sake of brevity, the condition and care of the children, preparation and composition of the diets and all details of experimental procedure, which were given in the first report of the two experiments (Hawks, Dye and Bray, '37) are omitted. The children, however, lived under extremely constant conditions for relatively long periods of time. The two children in the first experiment did not have exactly the same foods

as the three children in the second experiment, but they all had, first, a diet containing 3 and then one containing 4 gm. of protein per kilogram per day. All technical procedures were exactly the same. The calorie values of food, urine and feces, for 3-day collection periods, were determined by means of the oxy-calorimeter (Benedict and Fox, '25 a and '25 b).

## RESULTS AND DISCUSSION

In order to interpret the results of an experiment, it is important to know when the subjects reach an equilibrium and how much of the experimental data should be considered as preliminary. Therefore it was necessary to determine, first, whether the children were in calorie equilibrium when receiving the 3-gm. protein diet and second, the length of time necessary to re-establish equilibrium following the change to the 4-gm. protein diet. The data for the seven 3-day periods on the medium protein diet (table 1) collected following preliminary periods of 10 or 12 days, were extremely constant and showed that the children were undoubtedly in calorie equilibrium. The small period-to-period fluctuations in intake had little effect on urine and feces values, since, with the exception of the feces values for the children in the first experiment, all output figures varied within one calorie for each child and represented a relatively constant percentage of the intake calories. Statistical evaluation of the data showed coefficients of variation for urine and feces ranging from 5.6 to 12.8. Although relatively larger than those for intake, which were 1.2 and 1.3, they were not high. The absorption and retention values also indicated that the children were in calorie equilibrium. The coefficients of variation for absorption, retention and intake were practically identical for each experiment, being 1.5, 1.5 and 1.2, respectively, in experiment 1, and 3.5, 3.6 and 3.3 in experiment 2. These similar coefficients showed that absorption and retention values fluctuated to the same degree as intake figures. The high correlations between absorption and intake, 0.746 and 0.939 for experiment 1 and 2, respectively, and between retention and intake, 0.731 and

TABLE 1

*Calorie balance data on a constant 3-gm. protein diet, expressed per kilogram of body weight*

| CHILD | EXPERIMENT | PERIOD | INTAKE CALORIES | OUTPUT | | | | | | | ABSORPTION | | RETENTION | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Urine | | | Feces | | Total | | | | | |
| | | | | Total calories | Proportion intake | Ratio calories/nitrogen | Total calories | Proportion intake | Total calories | Proportion intake | Total calories | Proportion intake | Total calories | Proportion intake |
| | | | | | % | | | % | | % | | % | | % |
| B | I | 1 | 85.8 | 3.4 | 4.0 | 8.8 | 6.1 | 7.1 | 9.5 | 11.1 | 79.7 | 92.9 | 76.3 | 88.9 |
| | | 2 | 87.0 | 3.1 | 3.6 | 8.2 | 6.6 | 7.6 | 9.7 | 11.2 | 80.4 | 92.4 | 77.3 | 88.8 |
| | | 3 | 85.8 | 3.1 | 3.6 | 7.8 | 5.2 | 6.1 | 8.3 | 9.7 | 80.6 | 93.9 | 77.5 | 90.3 |
| | | 4 | 85.1 | 3.3 | 3.8 | 7.8 | 4.9 | 5.8 | 8.2 | 9.6 | 80.2 | 94.2 | 76.9 | 90.4 |
| | | 5 | 86.4 | 3.2 | 3.7 | 8.0 | 5.3 | 6.1 | 8.5 | 9.8 | 81.1 | 93.9 | 77.9 | 90.2 |
| | | 6 | 86.8 | 3.5 | 4.1 | 8.5 | 5.1 | 5.8 | 8.6 | 9.9 | 81.7 | 94.2 | 78.2 | 90.1 |
| | | 7 | 87.4 | 3.4 | 3.9 | 8.2 | 4.9 | 5.6 | 8.3 | 9.5 | 82.5 | 94.4 | 79.1 | 90.5 |
| | | Mean | 86.3 | 3.3 | 3.8 | 8.2 | 5.4 | 6.3 | 8.7 | 10.1 | 80.9 | 93.7 | 77.6 | 89.9 |
| D | I | 1 | 84.9 | 3.4 | 4.0 | 8.2 | 6.9 | 8.1 | 10.3 | 12.1 | 78.0 | 91.9 | 74.6 | 87.9 |
| | | 2 | 86.5 | 3.2 | 3.7 | 7.7 | 5.8 | 6.7 | 9.0 | 10.4 | 80.7 | 93.3 | 77.5 | 89.6 |
| | | 3 | 85.0 | 3.1 | 3.7 | 7.2 | 4.5 | 5.3 | 7.6 | 9.0 | 80.5 | 94.7 | 77.4 | 91.0 |
| | | 4 | 83.9 | 3.1 | 3.8 | 7.3 | 4.9 | 5.8 | 8.0 | 9.6 | 79.0 | 94.0 | 75.9 | 90.4 |
| | | 5 | 85.0 | 3.1 | 3.7 | 7.3 | 5.8 | 6.8 | 8.9 | 10.5 | 79.2 | 93.2 | 76.1 | 89.5 |
| | | 6 | 86.0 | 3.1 | 3.6 | 7.0 | 5.2 | 6.0 | 8.3 | 9.6 | 80.8 | 94.0 | 77.7 | 90.4 |
| | | 7 | 86.8 | 2.8 | 3.2 | 6.7 | 5.4 | 6.2 | 8.2 | 9.4 | 81.4 | 93.8 | 78.6 | 90.6 |
| | | Mean | 85.4 | 3.1 | 3.6 | 7.3 | 5.5 | 6.5 | 8.6 | 10.1 | 79.9 | 93.5 | 76.8 | 89.9 |
| V | II | 1 | 90.7 | 2.4 | 2.6 | 6.0 | 3.7 | 4.1 | 6.1 | 6.7 | 87.0 | 95.9 | 84.6 | 93.3 |
| | | 2 | 87.0 | 2.8 | 3.1 | 6.9 | 3.7 | 4.3 | 6.5 | 7.4 | 83.3 | 95.7 | 80.5 | 92.6 |
| | | 3 | — | — | — | — | — | — | — | — | — | — | — | — |
| | | 4 | — | — | — | — | — | — | — | — | — | — | — | — |
| | | 5 | 87.1 | 2.7 | 3.1 | 7.0 | 4.0 | 4.6 | 6.7 | 7.7 | 83.1 | 95.4 | 80.4 | 92.3 |
| | | 6 | 88.1 | 2.8 | 3.2 | 7.0 | 3.7 | 4.2 | 6.5 | 7.4 | 84.4 | 95.8 | 81.6 | 92.6 |
| | | 7 | 86.6 | 2.6 | 3.0 | 6.9 | 3.6 | 4.2 | 6.2 | 7.2 | 83.0 | 95.8 | 80.4 | 92.8 |
| | | Mean | 87.9 | 2.7 | 3.1 | 6.8 | 3.7 | 4.2 | 6.4 | 7.3 | 84.2 | 95.8 | 81.5 | 92.7 |
| C | II | 1 | 94.0 | 2.8 | 3.0 | 7.0 | 4.4 | 4.7 | 7.2 | 7.7 | 89.6 | 95.3 | 86.8 | 92.3 |
| | | 2 | 90.6 | 2.6 | 2.9 | 6.6 | 4.5 | 5.0 | 7.1 | 7.9 | 86.1 | 95.0 | 83.5 | 92.1 |
| | | 3 | 91.6 | 2.8 | 3.1 | 6.9 | 4.4 | 4.8 | 7.2 | 7.9 | 87.1 | 95.2 | 84.4 | 92.1 |
| | | 4 | 92.2 | 3.1 | 3.4 | 7.6 | 4.4 | 4.8 | 7.5 | 8.2 | 87.8 | 95.2 | 84.7 | 91.8 |
| | | 5 | 90.4 | 3.0 | 3.3 | 7.6 | 4.5 | 5.0 | 7.5 | 8.3 | 85.9 | 95.0 | 82.9 | 91.7 |
| | | 6 | 91.5 | 2.8 | 3.0 | 6.9 | 4.3 | 4.7 | 7.1 | 7.7 | 87.2 | 95.3 | 84.4 | 92.3 |
| | | 7 | 89.8 | 2.8 | 3.1 | 7.4 | 4.2 | 4.7 | 7.0 | 7.8 | 85.6 | 95.3 | 82.8 | 92.2 |
| | | Mean | 91.4 | 2.8 | 3.1 | 7.1 | 4.4 | 4.8 | 7.2 | 7.9 | 87.0 | 95.2 | 84.2 | 92.1 |
| J | II | 1 | 88.0 | 2.6 | 2.9 | 7.2 | 4.2 | 4.8 | 6.8 | 7.7 | 83.8 | 95.2 | 81.3 | 92.3 |
| | | 2 | 84.6 | 2.5 | 3.0 | 6.9 | 4.6 | 5.4 | 7.1 | 8.4 | 80.0 | 94.6 | 77.5 | 91.6 |
| | | 3 | 85.5 | 2.6 | 3.1 | 7.2 | 4.6 | 5.4 | 7.2 | 8.5 | 80.9 | 94.6 | 78.3 | 91.5 |
| | | 4 | 86.8 | 2.7 | 3.1 | 7.3 | 4.9 | 5.6 | 7.6 | 8.7 | 81.9 | 94.4 | 79.2 | 91.3 |
| | | 5 | 85.1 | 2.7 | 3.2 | 7.3 | 4.9 | 5.8 | 7.6 | 9.0 | 80.2 | 94.2 | 77.5 | 91.0 |
| | | 6 | 85.9 | 2.7 | 3.1 | 7.3 | 4.5 | 5.2 | 7.2 | 8.3 | 81.4 | 94.8 | 78.7 | 91.7 |
| | | 7 | 84.1 | 2.7 | 3.2 | 7.8 | 4.5 | 5.3 | 7.2 | 8.5 | 79.6 | 94.7 | 76.9 | 91.5 |
| | | Mean | 85.7 | 2.6 | 3.1 | 7.3 | 4.6 | 5.4 | 7.2 | 8.4 | 81.1 | 94.6 | 78.5 | 91.6 |

0.936 in the two experiments, as well as the constant percentages of the intake absorbed and retained (table 1) indicated that all of these values fluctuated in the same manner.

The change to the 4-gm. protein diets, which immediately followed use of the medium protein diets, did not disturb the calorie equilibrium (table 2). In the study of nitrogen balances, Hawks, Bray and Dye ('38) found that the values fluctuated more during the first periods following the change in diets than in succeeding periods, and in the study of the variation in the urinary nitrogenous constituents ('37), they reported more variable data during the first three periods on the high protein diets. In the present study on calorie balances there seemed to be no consistent differences between the values for the first periods following the change in diets and those for the subsequent periods, and there were no significant differences between the mean values of the data for all of the periods together, for the first three periods or for periods four to eight. Statistical evaluation of the data also showed that there was no need for a preliminary period. Both the standard deviations and the coefficients of variation for all output figures and for absorption and retention values were practically the same for the last five periods and for the total values representing all periods. Although preliminary periods may not have been necessary in this study, it does not follow that they could be omitted from all calorie balance studies. If the calories or other factors varied to a great extent, preliminary periods might be necessary.

Although the diets used in the two experiments were practically the same, there were some differences in the results, which can be seen by comparing the mean values of the results obtained in the first experiment with those obtained in the second. Table 1 shows that the percentages of the intake excreted and retained were almost identical for the children within each experiment, but that the results for the two experiments differed. For example, the two children in the first experiment excreted identical percentages of the calorie intake, but a larger percentage than the children in the second

TABLE 2

*Calorie balance data on a constant 4-gm. protein diet, expressed per kilogram of body weight*

| CHILD | EXPERIMENT | PERIOD | INTAKE CALORIES | OUTPUT | | | | | | | ABSORPTION | | RETENTION | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Urine | | | Feces | | Total | | | | | |
| | | | | Total calories | Proportion intake | Ratio calories/nitrogen | Total calories | Proportion intake | Total calories | Proportion intake | Total calories | Proportion intake | Total calories | Proportion intake |
| | | | | | % | | | % | | % | | % | | % |
| B | I | 1 | 92.4 | 4.3 | 4.6 | 8.5 | 7.3 | 7.9 | 11.6 | 12.5 | 85.1 | 92.1 | 80.8 | 87.5 |
| | | 2 | 89.7 | 4.8 | 5.3 | 8.5 | 7.0 | 7.8 | 11.8 | 13.1 | 82.7 | 92.2 | 77.9 | 86.9 |
| | | 3 | 93.0 | 4.3 | 4.6 | 8.1 | 6.7 | 7.2 | 11.0 | 11.8 | 86.3 | 92.8 | 82.0 | 88.2 |
| | | 4 | 90.4 | 4.1 | 4.5 | 7.5 | 5.1 | 5.6 | 9.2 | 10.1 | 85.2 | 94.4 | 81.2 | 89.9 |
| | | 5 | 87.9 | 5.0 | 5.7 | 8.6 | 5.9 | 6.7 | 10.9 | 12.4 | 82.0 | 93.3 | 77.0 | 87.6 |
| | Mean | 1–3 | 91.7 | 4.4 | 4.8 | 8.4 | 7.0 | 7.6 | 11.4 | 12.4 | 84.7 | 92.4 | 80.3 | 87.6 |
| | Mean | All | 90.7 | 4.5 | 5.0 | 8.2 | 6.4 | 7.0 | 10.9 | 12.0 | 84.3 | 93.0 | 79.8 | 88.0 |
| D | I | 1 | 91.3 | 4.0 | 4.4 | 7.4 | 6.0 | 6.6 | 10.0 | 11.0 | 85.3 | 93.4 | 81.3 | 89.0 |
| | | 2 | 88.7 | 4.2 | 4.7 | 7.1 | 5.5 | 6.2 | 9.7 | 10.9 | 83.2 | 93.8 | 79.0 | 89.1 |
| | | 3 | 92.5 | 4.3 | 4.6 | 7.5 | 5.8 | 6.3 | 10.1 | 10.9 | 86.7 | 93.7 | 82.4 | 89.1 |
| | | 4 | 89.6 | 4.2 | 4.7 | 7.4 | 5.1 | 5.7 | 9.3 | 10.4 | 84.5 | 94.3 | 80.3 | 89.6 |
| | | 5 | 87.2 | 4.1 | 4.7 | 7.1 | 5.2 | 6.0 | 9.3 | 10.7 | 82.0 | 94.0 | 77.9 | 89.3 |
| | Mean | 1–3 | 90.8 | 4.2 | 4.6 | 7.3 | 5.7 | 6.3 | 9.9 | 10.9 | 85.1 | 93.7 | 80.9 | 89.1 |
| | Mean | All | 89.9 | 4.2 | 4.7 | 7.3 | 5.5 | 6.1 | 9.7 | 10.8 | 84.4 | 93.9 | 80.2 | 89.2 |
| V | II | 1 | 87.5 | 3.6 | 4.1 | 7.1 | 3.7 | 4.2 | 7.3 | 8.3 | 83.8 | 95.8 | 80.2 | 91.7 |
| | | 2 | 88.3 | 3.6 | 4.1 | 6.6 | 4.1 | 4.6 | 7.7 | 8.7 | 84.2 | 95.4 | 80.6 | 91.3 |
| | | 3 | 87.1 | 3.7 | 4.2 | 6.8 | 4.0 | 4.6 | 7.7 | 8.8 | 83.1 | 95.4 | 79.4 | 91.2 |
| | | 4 | 90.6 | 3.8 | 4.2 | 6.9 | 4.6 | 5.1 | 8.4 | 9.3 | 86.0 | 94.9 | 82.2 | 90.7 |
| | | 5 | 86.3 | 3.5 | 4.0 | 6.6 | 4.1 | 4.8 | 7.6 | 8.8 | 82.2 | 95.2 | 78.7 | 91.2 |
| | | 6 | 88.6 | 3.5 | 4.0 | 6.6 | 3.9 | 4.4 | 7.4 | 8.4 | 84.7 | 95.6 | 81.2 | 91.6 |
| | | 7 | 85.6 | 3.7 | 4.4 | 6.7 | 3.8 | 4.4 | 7.5 | 8.8 | 81.8 | 95.6 | 78.1 | 91.2 |
| | | 8 | 89.4 | 3.6 | 4.0 | 6.7 | 3.6 | 4.0 | 7.2 | 8.0 | 85.8 | 96.0 | 82.2 | 92.0 |
| | Mean | 1–3 | 87.6 | 3.7 | 4.2 | 6.8 | 3.9 | 4.5 | 7.6 | 8.7 | 83.7 | 95.5 | 80.0 | 91.3 |
| | Mean | 4–8 | 88.1 | 3.6 | 4.1 | 6.7 | 4.0 | 4.5 | 7.6 | 8.6 | 84.1 | 95.5 | 80.5 | 91.4 |
| | Mean | All | 87.9 | 3.6 | 4.1 | 6.8 | 4.0 | 4.5 | 7.6 | 8.6 | 83.9 | 95.5 | 80.3 | 91.4 |
| C | II | 1 | 90.5 | 3.6 | 4.0 | 7.1 | 4.8 | 5.3 | 8.4 | 9.3 | 85.7 | 94.7 | 82.1 | 90.7 |
| | | 2 | 91.1 | 4.0 | 4.4 | 7.2 | 3.7 | 4.1 | 7.7 | 8.5 | 87.4 | 95.9 | 83.4 | 91.5 |
| | | 3 | 89.4 | 3.7 | 4.1 | 6.7 | 4.6 | 5.2 | 8.3 | 9.3 | 84.8 | 94.8 | 81.1 | 90.7 |
| | | 4 | 92.7 | 3.7 | 4.0 | 6.8 | 5.0 | 5.4 | 8.7 | 9.4 | 87.7 | 94.6 | 84.0 | 90.6 |
| | | 5 | 88.5 | 3.8 | 4.3 | 7.1 | 4.7 | 5.3 | 8.5 | 9.6 | 83.8 | 94.7 | 80.0 | 90.4 |
| | | 6 | 91.3 | 3.8 | 4.2 | 7.1 | 4.6 | 5.0 | 8.4 | 9.2 | 86.7 | 95.0 | 82.9 | 90.8 |
| | | 7 | 87.4 | 3.7 | 4.2 | 6.8 | 4.2 | 4.8 | 7.9 | 9.0 | 83.2 | 95.2 | 79.5 | 91.0 |
| | | 8 | 91.5 | 3.7 | 4.0 | 6.6 | 4.1 | 4.5 | 7.8 | 8.5 | 87.4 | 95.5 | 83.7 | 91.5 |
| | Mean | 1–3 | 90.3 | 3.7 | 4.1 | 7.0 | 4.4 | 4.9 | 8.1 | 9.0 | 85.9 | 95.1 | 82.2 | 91.0 |
| | Mean | 4–8 | 90.3 | 3.8 | 4.2 | 6.9 | 4.5 | 5.0 | 8.3 | 9.2 | 85.8 | 95.0 | 82.0 | 90.8 |
| | Mean | All | 90.3 | 3.7 | 4.1 | 6.9 | 4.5 | 5.0 | 8.2 | 9.1 | 85.8 | 95.0 | 82.1 | 90.9 |
| J | II | 1 | 85.1 | 3.2 | 3.8 | 6.7 | 4.6 | 5.4 | 7.8 | 9.2 | 80.5 | 94.6 | 77.3 | 90.8 |
| | | 2 | 85.7 | 3.7 | 4.3 | 7.0 | 4.4 | 5.1 | 8.1 | 9.4 | 81.3 | 94.9 | 77.6 | 90.6 |
| | | 3 | 83.6 | 3.3 | 3.9 | 6.6 | 4.6 | 5.5 | 7.9 | 9.4 | 79.0 | 94.5 | 75.7 | 90.6 |
| | | 4 | 86.5 | 3.9 | 4.5 | 7.9 | 4.3 | 5.0 | 8.2 | 9.5 | 82.2 | 95.0 | 78.3 | 90.5 |
| | | 5 | 82.8 | 3.5 | 4.2 | 7.1 | 4.6 | 5.6 | 8.1 | 9.8 | 78.2 | 94.4 | 74.7 | 90.2 |
| | | 6 | 85.4 | 3.4 | 4.0 | 6.9 | 4.6 | 5.4 | 8.0 | 9.4 | 80.8 | 94.6 | 77.4 | 90.6 |
| | | 7 | 82.0 | 3.4 | 4.2 | 6.8 | 4.6 | 5.6 | 8.0 | 9.8 | 77.4 | 94.4 | 74.0 | 90.2 |
| | | 8 | 85.6 | 3.2 | 3.7 | 6.5 | 4.6 | 5.4 | 7.8 | 9.1 | 81.0 | 94.6 | 77.8 | 90.9 |
| | Mean | 1–3 | 84.8 | 3.4 | 4.0 | 6.8 | 4.5 | 5.3 | 7.9 | 9.3 | 80.3 | 94.7 | 76.9 | 90.7 |
| | Mean | 4–8 | 84.5 | 3.5 | 4.1 | 7.0 | 4.6 | 5.4 | 8.1 | 9.5 | 79.9 | 94.6 | 76.4 | 90.5 |
| | Mean | All | 84.6 | 3.4 | 4.1 | 6.9 | 4.6 | 5.4 | 8.0 | 9.5 | 80.0 | 94.6 | 76.6 | 90.5 |

experiment, who had, therefore, more available calories. There may have been many reasons for the differences in the results of the two experiments, but the slight variations in the composition of the diets was probably the main reason. Therefore, the results of the two experiments could not be averaged, since erroneous conclusions might result.

In attempting to find the effect of diet on calorie balance, it seemed wise to base the discussion entirely on the results found in the second experiment, because there were fewer variables in the diets used here. The average calorie values in both diets were identical and the constituents of the diets were also the same, except for slight changes in the second one, made to increase the protein content and to keep the calories at the same level (Hawks, Dye and Bray, '37)[4]

To determine whether or not the period-to-period fluctuations in diet calories influenced the number of calories retained, averages of the intake and retention values per kilogram for all of the children, for each separate period, were calculated. This was possible because the children received exactly the same foods and the same number of calories per kilogram. A comparison of these averages (fig. 1)[5] showed that the small diet fluctuations were reflected in the retention values and that even the change to the high protein diet did not influence this relationship. The constant percentage of the intake calories retained during the several periods on each diet (tables 1 and 2), as well as the high correlations between intake and retention (0.936 on the 3-gm. and 0.940 on the 4-gm. protein diets), also substantiated this relationship. Therefore, the number of available calories would seem to be dependent upon the intake calories.

Nevertheless, the change to the high protein diet also affected the calorie retention. The percentages of the intake retained were lower then those on the medium protein diet

[4] The only differences in the 4-gm. protein diet were the addition of egg white and gelatin and the omission of a portion of the butter.

[5] Although the discussion includes only the results of experiment 2, the figure gives the data for both experiments.

(table 2) because the high protein diet increased the total number of calories eliminated, both in urine and feces. These increases were not large, but the urine values were 1% and the feces values from 0.0 to 0.3% higher. It is probable that the increased nitrogen eliminated was responsible for almost

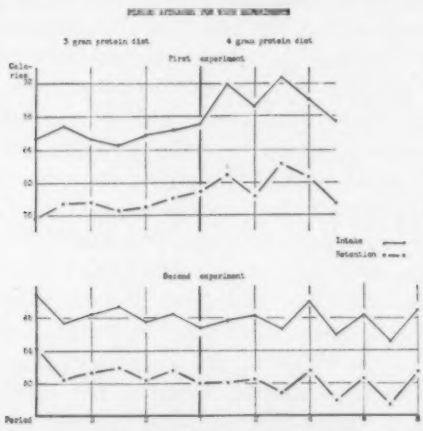


**Fig. 1** The relationship between the intake and retention values obtained by averaging the per kilogram data for all of the children for each separate period.

TABLE 3

*Increase in calorie values per kilogram on high protein diet as analyzed and as calculated from nitrogenous end products*

| CHILD | URINE CALORIES | | FECES CALORIES | |
|---|---|---|---|---|
| | Analyzed | Calculated | Analyzed | Calculated |
| V | 0.9 | 0.915 | 0.3 | 0.250 |
| C | 0.9 | 0.896 | 0.1 | 0.125 |
| J | 0.8 | 0.777 | 0.0 | 0.075 |

all of the increased calorie values in the excreta. Several facts substantiate this. The first is, that the increases in the values for analyzed calories and for those calculated (table 3) from the nitrogenous end products (Hawks, Bray and Dye, '37) were practically the same. Second, the calories excreted in the urine seemed to depend upon the nitrogen in the diet, since the correlation between these values was 0.932. Third,

the average ratios between urinary calories and nitrogen were practically the same for each child on the two diets (tables 1 and 2). Fourth, the correlations between the values for fecal calories and the dry weight of the feces, which the increase in nitrogenous end products probably caused, were 0.917 on both diets.[6] Therefore, all of the above facts seem to indicate that nitrogen may have been the sole cause for the increased excretory calories on the high protein diet.

The increased proportion of the calories excreted on the high protein diet indicated that a definite proportion of calories was not always eliminated in the excreta. In the present experiment, the average percentage excreted in urine and feces varied from 7.3 to 12.0. This difference of nearly 5% shows the necessity for actual analyses of the excretory material for most accurate results, since the practice of either subtracting 10% from the intake values, or of using physiological values, may introduce considerable error.

The higher proportional output of calories on the high protein diet caused a decrease in the number of calories retained, or a decrease in the number available for basal metabolic needs, growth and activity. Nevertheless, the children gained weight at a more rapid rate during this period than they had on the medium protein diet, when they had had a larger number of available calories (table 4). Although this seemed impossible, the results conformed with those which Forbes et al. ('35, '38 and '39) obtained for rats in a similar experiment. They fed equicaloric diets which contained protein in amounts varying from 10 to 45%. As the protein content of the diets increased, the metabolizable energy, or total calories retained, decreased. Even with the lowered available energy, the rate of weight gains was progressively greater, until the diet contained 25% protein. Above that level, the weight gain of the

[6] If the increased nitrogen in the feces caused the entire calorie increase, other constituents of the diet besides nitrogen must have been eliminated in relatively constant amounts. This fact adds some evidence to the contention, discussed in connection with the nitrogen data (Hawks, Bray and Dye, '38), that there is a constant fraction eliminated in the feces.

rats diminished. The diets of the children in the present experiment contained comparable percentages of protein, approximately 13 and 18% for the 3- and 4-gm. protein diets respectively. Therefore, under similar conditions, both rats and children gained at an increased rate with increase in the protein content of the diet.

It is impossible to tell from the present experiment whether the reduction in the available calories gave fewer calories for growth, produced a lower basal metabolism, or curtailed activity. Forbes and his associates suggested that the activity of their rats might have been reduced, because calculated heat production, which represents basal metabolism plus activity, became lower as the protein in the diet increased and at the same time basal metabolism values remained constant. Since several investigators (Wang and her associates, '30; DuBois, '36), have reported that the protein content of the human diet does not influence the basal metabolism, it may be that these same facts are true for the children in the present study. As far as could be determined, however, the activity of the children remained the same on the two diets, since they spent approximately the same time each day in active exercise and followed a regular routine.

TABLE 4

*Average gain in weight per day*

| DIET | B | D | V | C | J |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Medium protein | 1.43 | 0.95 | 3.81 | 4.29 | 2.38 |
| High protein | 5.33 | 4.67 | 4.58 | 8.75 | 8.75 |

There may be several reasons for the increase in the weight gains of the children on the high protein diet. Since the diet increased the coefficient of digestibility of nitrogen and the fraction of the intake absorbed was raised as much as 2.6% (Hawks, Bray and Dye, '38), it may be possible that the egg white and gelatin used to increase the nitrogen content of the diet were more effective for producing weight gains than calories from other sources, even if the actual number of

calories absorbed was lower. The storage of a larger number of grams of protein may also have increased the water content of the body and thus increased the weight gains. These facts may indicate that 3 gm. of protein per kilogram of body weight may not be sufficient for optimum growth as well as for optimum protein storage. Judging from the work of Forbes et al., even a 4-gm. protein diet may not contain enough protein to produce the most efficient utilization. More work on these subjects, however, would have to be done before definite conclusions could be drawn.

Although the higher protein content of the diet did produce changes in calorie balances, it seemed to cause no differences in the variability of the calorie data. The urine and feces values remained strikingly constant from period to period, as they had on the medium protein diet. The absorption and retention values fluctuated to the same degree and in the same manner, as indicated by the high correlations with intake, 0.939 and 0.940, for absorption and retention respectively.

Thus it may be said that both calories and protein influenced calorie utilization. The availability seemed to be directly proportional to calorie fluctuations in diet, irrespective of the change in the protein content, but at the same time the increase in protein reduced the percentage of the intake calories absorbed and retained.

## SUMMARY

1. Five pre-school children served as subjects for two long-time balance experiments, in each of which they received two diets, the first containing 3 and the second 4 gm. of protein per kilogram of body weight.

2. The increase in the protein content of the diets did not change the constant level of calorie utilization. Therefore, preliminary periods before the high protein diets seemed unnecessary in these experiments.

3. The children in each experiment reacted in the same manner, although there were slight differences in the results of the two experiments.

4. The period-to-period variations for excretory values remained exceedingly constant on both diets and the absorption and retention figures varied in the same manner and to the same degree as the intake values.

5. The change from the 3- to the 4-gm. protein diet affected calorie balance as follows: (a) It increased the nitrogen content of the excreta, thus increasing the actual number of calories eliminated. (b) It increased the average proportion of the intake calories eliminated from 7.3 to 12.0%. Thus, subtracting 10% from the intake values to care for excretory losses does not always give accurate results. (c) It reduced the actual number, as well as the percentage, of the intake calories available for body needs, but at the same time it produced greater weight gains in the children.

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# MINIMUM VITAMIN A AND CAROTENE REQUIREMENTS OF MAMMALIAN SPECIES [1]

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ONE FIGURE

(Received for publication September 5, 1939)

This paper presents data on the vitamin A and carotene requirements of the horse, re-evaluates and summarizes previous data from this laboratory and correlates all this material with other information pertinent to the requirements of mammalia in general.

## EXPERIMENTAL PART

The methods of procedure were similar to those used in the experiments with cattle (Guilbert and Hart, '35) and cattle, sheep, and swine (Guilbert, Miller and Hughes, '37).

### *Rations*

The basal rations used for the nine horses upon which we have quantitative data are shown in table 1. Barley straw was used for roughage during the depletion period for horses nos. 1, 2, 3, and 4. During the later part of the experimental period for horse no. 3, straw was omitted from the ration. No carotene analyses were made on the concentrate mixtures or straw during the period when the animals' vitamin-A stores were being depleted. During the experimental periods when carotene or vitamin-A requirements were being determined, com-

[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

posite samples were analyzed and total carotene intake was computed on the basis of both the supplement and the basal ration. It was found that the animals thrived without the addition of roughage, and to lower the carotene content of the ration, straw was omitted in later experiments. All of the animals chewed on fences and mangers, which necessitated their protection with wire and metal strips. Omission of roughage may have increased this activity. Except for the substitution of straw for hay, the rations of the first four animals were typical of widely-used horse rations.

TABLE 1

*Basal rations*

| FEEDS | HORSE NUMBER | | | | |
|---|---|---|---|---|---|
| | 1 and 2 | 3 and 4 | 5 | 7 and 8 | 10 and 12 |
| Dried molasses beet pulp | .. | .. | .. | 55 | 53.5 |
| Rolled barley | 30 | 30 | 30 | 10 | 10 |
| Rolled oats | 30 | 30 | 30 | 10 | 10 |
| Wheat bran | 30 | 30 | 30 | 10 | 10 |
| Linseed oil meal | 10 | 8.5 | 5 | 10 | 10 |
| Cottonseed meal | .. | .. | 5 | 5 | 5 |
| $CaCO_3$ | .. | 1.5 | .. | .. | 1.5 |
| Straw | ad lib. | ad lib. | .. | .. | .. |

## *Depletion time*

The purebred Percherons used were raised on the University Farm. Previous to being placed on experiment, they had had normal rations including abundant alfalfa hay and pasture.

The age of animals nos. 1 to 4 inclusive when started on experiment varied from 284 to 376 days and the time elapsing before the onset of night blindness varied from 492 to 627 days. The depletion time for nos. 5 to 12 inclusive was significantly less than for the first four animals, presumably because their basal rations without straw contained less carotene. The age of these animals when placed on experiment varied from 119 to 444 days and the depletion time varied from 265 to 439 days.

*Symptoms*

The first detectable symptom in the horse, similar to that of other species, was night blindness. The horse normally appears to have particularly good night vision and has an acute sense of direction and memory for the position of obstacles. This latter trait frequently made it difficult to ascertain whether or not animals could see when they were in quarters to which they were accustomed. Moreover, once they had run into obstacles they were cautious to avoid repeating their mistakes, thus making routine testing for night blindness more difficult than with cattle, sheep, and swine. A series of movable panels set up in an alley-way adjoining the pens formed a varying maze that permitted us to judge more critically visual acuity. Tests generally were made when black 144 point Ebar light condensed capital letters on white background were discernible at 3 to 6 feet.

When completely night blind, the animals carried their heads high, with nose extended, or close to the ground, feeling their way around. They moved rather freely, however, in their own lots. If the gate into the alley was opened, the horses were driven through only with difficulty. Time after time they could be driven at a trot up to the normal position of the gate, at which point they stopped abruptly, whirled, always just missing the normal position of the gate. This strikingly demonstrated their uncanny memory for position of obstacles as well as their complete inability to see in light that plainly revealed surrounding objects to normal persons and horses. Once they were placed outside their lot and forced by driving to move rapidly, they ran head on into panels placed in their path. They often became completely lost and greatly excited. Partially defective vision could be distinguished by the distance at which they were able to see obstacles and alter their course to avoid collision.

If vitamin-A therapy were not instituted after the appearance of night blindness, symptoms progressed gradually over a period of 3 to 4 months. The hair became rough, and later

there was clouding of the corneas and impaired daylight vision. Excessive lachrymation rather than xerophthalmia was exhibited. At this stage the eyes of nos. 2 and 3 were examined by Dr. D. K. Mills, ophthalmologist of the Woodland Clinic. The change in the corneas was found to be primarily in the deeper layers rather than on the surface. This keratinization appeared first as streaks and later appeared to involve equally the whole cornea. Comparison of the eyes at this stage with those of normal animals revealed no swelling nor elevation of the disc. The horses observed did not show the susceptibility to convulsions that is exhibited by cattle. On one occasion, however, no. 2 staggered and went down just prior to the onset of estrus. Vitamin-A deficient horses did show some nervousness and increased irritability when touched. No. 3, a stallion, bred filly no. 2 several times when both were showing night blindness. This confirms other observations that early stages of deficiency do not affect estrus or libido. We have no record of impregnation, however, under these conditions.

Horse no. 3 at about the onset of corneal involvement developed a nasal discharge and snoring, which became progressively worse as the deficiency advanced. The appetite declined and the animal, after losing over 100 pounds weight, became weak. Ten days after giving small doses (5 cc. daily of cod liver oil containing 2600 I.U. per gram), the corneas were clear and the eyes stopped watering; breathing became easier; appetite and general condition rapidly improved. Night vision became normal within 30 days. The snoring never entirely disappeared. After cod liver oil feeding was stopped, the same general sequence of symptoms reappeared. Snoring became worse and breathing difficult. Six weeks after cessation of vitamin A therapy breathing became very difficult. The animal suddenly began bleeding and discharging from the nostrils, went down, and did not get up for several hours. Later he got up without help and breathing was easier. Diarrhea developed during the next week, appetite declined, the animal became weaker and a month later developed acute difficulty in breath-

ing and died. Post mortem examination revealed no obstruction in the nasal passages.

Animal no. 2 gradually became weak, and finally, unable to rise, was killed in extremis. Animal no. 4 developed night blindness during the last month of gestation, had difficulty in labor, and died following removal of a dead foal that was abnormally presented. Horse no. 1 became so lame from bone and joint abnormalities that he could not get up and was killed. This animal was receiving cod liver oil prior to autopsy and had some vitamin-A storage. The other animals gave negative tests for vitamin A in the liver. Animal nos. 5 to 12 inclusive were still living at the time of writing.

### MINIMUM VITAMIN A AND CAROTENE REQUIREMENTS

Dehydrated alfalfa meal was used as a source of carotene. The methods of sampling, feeding, and analysis were the same as reported by Guilbert, Miller and Hughes ('37). The cod liver oil used was also from the same batch used in their experiments. Goss and Guilbert ('39) showed that no change had occurred in the extinction coefficient of this oil which had been stored at —10°C. They re-evaluated the spectrophotometric analysis on the basis of the extinction coefficient $E^{1\%}_{1\ cm}$ 328 mμ = 2100 found by Holmes and Corbet ('37) for crystalline vitamin A. Parallel rat experiments with this oil and U.S.P. Reference cod liver oil showed that it contained 2600 I.U. per gram, an amount slightly less than that indicated by comparative spectrophotometric analyses of the two oils.

When quantitative dosage was begun after the onset of night blindness carotene analyses were run at frequent intervals on composite samples of both straw and concentrate mixtures. The straw contained more carotene than that used in previous experiments and varied from 0.05 to 0.10 mg. %. The concentrate mixtures consistently contained between 0.01 and 0.02 mg. % of pigment measured colorimetrically as carotene. These amounts in the basal rations including straw, contributed between 5 and 10 micrograms of carotene per kilogram body weight as shown in table 2.

TABLE 2

*Minimum carotene and vitamin-A requirements of the horse (source of carotene, alfalfa meal; source of vitamin A, cod liver oil)*

| ANIMAL NUMBER | DURATION OF PERIOD | INTAKE | RESULTS |
|---|---|---|---|
| | *days* | *μg. per kg.* | |
| | .. | Carotene 5 to 10 [1] | Became night blind |
| 1 | 14 | Carotene 29 to 33 | Vision became normal |
| | 60 | Carotene 19 to 26 | Vision borderline to normal |
| | .. | Carotene 5 to 10 [1] | Became night blind |
| | 22 | Carotene 30 to 35 | Vision became normal |
| 2 | 60 | Carotene 20 to 26 | Vision remained normal |
| | 41 | Carotene 5 to 10 [1] | Became night blind |
| | 27 | Carotene 18 to 24 | Partially night blind |
| | .. | Carotene 5 to 10 [1] | Became night blind |
| 3 | 27 | Carotene 15 to 23 | Partially night blind |
| | 20 | Carotene 25 to 30 | Vision became normal |
| | 27 | Carotene 5 to 10 [1] | Became night blind |
| | .. | Carotene 5 to 10 [1] | Became night blind |
| 4 | 32 | Carotene 18 to 23 | Vision became normal |
| | 42 | Carotene 5 to 10 [1] | Became night blind |
| | 41 | Vitamin A 1.5 [1] | Became night blind |
| 2 | 59 | Vitamin A 5.1 | Vision became normal |
| | 41 | Vitamin A 1.5 [1] | Became night blind |
| | 27 | Vitamin A 1.5 [1] | Became night blind |
| | 41 | Vitamin A 7.3 | Vision became normal in 27 days |
| | 145 | Vitamin A 1.5 [1] | Became night blind in 73 days |
| 3 | 124 | Vitamin A 0.4 [1] | Advanced symptoms developed |
| | 72 | Vitamin A 5.3–5.8 | Marked improvement, sight normal |
| | 30 | Vitamin A 0.4 [1] | Became night blind |
| 5 | 31 | Vitamin A 4.2 | Vision became normal |
| | 11 | Vitamin A 0.4 [1] | Became night blind |
| 7 | 15 | Vitamin A 4.5 | Vision became normal |
| | 32 | Vitamin A 3.8 | Vision became normal |
| | 55 | Vitamin A 3.3 | Became partially night blind |
| | 30 | Vitamin A 3.7 | Vision remained partially defective |
| 8 | 10 | Vitamin A 4.1 | Vision remained slightly defective |
| | 9 | Vitamin A 0.4 [1] | Became completely night blind |
| | 21 | Vitamin A 4.3 | Remained partially night blind |
| | 32 | Vitamin A 5.3 | Vision became normal |
| | 8 | Vitamin A 3.7 | Vision became normal |
| | 59 | Vitamin A 3.0 | Borderline to normal vision |
| 10 | 70 | Vitamin A 0.4 [1] | Slowly became night blind |
| | 11 | Vitamin A 4.4 | Vision became normal |
| | 11 | Vitamin A 0.4 [1] | Became night blind |
| 12 | 17 | Vitamin A 4.3 | Vision became normal |

[1] Furnished by basal ration and straw.

In the experiments on vitamin-A requirement, the basal ration with straw was used only with animal no. 2 and the first trial with no. 3. In all other tests, straw was omitted. Since previous experiments have shown that the amount of carotene to meet minimum requirements is approximately five times that of vitamin A, the vitamin-A equivalent of the carotene furnished by the basal ration was computed on this basis. The average food consumption was about the same with respect to the body weight for the various animals. The average vitamin-A equivalent of the carotene in the basal ration with straw and without straw was roughly 1.5 and 0.4 microgram per kilogram body weight respectively as shown in table 2. The figure for vitamin-A intake during each test period includes the calculated vitamin-A equivalent of the carotene in the basal ration.

The adequate and subminimum carotene levels shown in table 2 overlap somewhat, largely because the extreme range of carotene contained in the basal ration was used in reporting the data. It is evident, however, that the minimum level lies between 20 and 30 micrograms per kilogram body weight which corresponds closely with previous results on other species.

In some instances individuals progressed very slowly from the first evidence of impaired vision to complete night blindness. The first trials with cod liver oil on nos. 8 and 10 may have been started while vestiges of reserves were being slowly withdrawn from fat or liver, as the dosage required to restore normal vision was less than that found with the same animals in later trials. With these exceptions, table 2 shows that vitamin-A levels less than 4 micrograms per kilogram body weight were borderline and that the adequate level lay between 4.2 and 5.3 micrograms per kilogram body weight.

It is significant that the higher level supplied by 5 cc. of cod liver oil containing 0.063% vitamin A as measured by the Hilger vitameter or 2600 I.U. per gram as compared with U.S.P. reference cod liver oil restored animal no. 3 to thrifty condition after he had progressed to a critical condition on

the basal ration. The weight of this animal varied between 1060 and 1165 pounds during the period.

## DISCUSSION

The same batch of cod liver oil was used in all of our experiments. It was kept at low temperature and analyses run from time to time revealed no change in its value. For the cattle, sheep, and swine experiments, it was analyzed by means of a Hilger Vitameter using the value $E^{1\%}_{1\ cm}$ 328 mμ = 1600. In order to compare these data with those found for horses and rats they have been re-evaluated on the basis of $E^{1\%}_{1\ cm}$ 328 mμ = 2100 reported for crystalline vitamin A (Holmes and Corbet, '37). The rat experiments of Goss and Guilbert ('39) compared the biological activity of this cod liver oil with that of U.S.P. Reference cod liver oil. This enables us also to express the requirement for vitamin A in international units. The data expressed both by weight and in international units are summarized in table 3.

TABLE 3

*Summary of data from this laboratory on minimum vitamin-A and carotene requirements of various species*

| SPECIES | DAILY INTAKE PER KILOGRAM BODY WEIGHT | | | |
|---|---|---|---|---|
| | Vitamin A | | Carotene | |
| | *μg.* | *I.U.* | *μg.* | *I.U.* |
| Cattle | 5.1–6.4 | 21–27 | 26–33 | 43–55 |
| Sheep | 4.3–6.3 | 17–26 | 25–35 | 42–58 |
| Swine | 4.4–5.7 | 18–24 | 25–39 | 42–65 |
| Horse | 4.2–5.3 | 17–22 | 20–30 | 33–50 |
| Rat | 4.6–5.3 [1] | .... | 15–20 | 25–33 |
| Rat | 3.8–4.6 [2] | 18–22 | .... | .... |

[1] Data based on cod liver oil that was also used in cattle, sheep, swine and horse experiments.

[2] Data based on U.S.P. Reference cod liver oil.

The minimum requirement for cattle, sheep, swine, and horses was defined as the lowest level per unit of body weight that prevented any detectable degree of nyctalopia under light conditions as described in this paper. The minimal level for

the rat was that which just sufficed to prevent any abnormal degree of cornification of the vaginal smears. In all cases, these minimum levels provided for normal growth and general well-being but permitted little or no storage. It is significant that with the same cod liver oil as the source of vitamin-A, the data for the rat and for the other species agree though different criteria of sufficiency were used. Since no significant storage occurred in any case, it is evident that the onset of night blindness and of vaginal cornification occur at the same level of deficiency.

The rat experiments showed that the U.S.P. Reference oil possessed slightly greater vitamin-A potency although both oils happened to have equal values as measured by the vitameter. We assume from this that the Reference oil contained less irrelevant material that contributed to light absorption at the vitamin-A wave length.

The minimum carotene level for the rat was slightly less than that found for other species, possibly due to more efficient absorption from the intestinal tract. These levels for the rat, however, varied from sub-minimum to adequate while all levels of dosage given for other species prevented night blindness. The lowest level in these cases may be taken as most nearly representing the actual minimum.

These data are in such good agreement that it is desirable to explore the possibility of similar consistent relationships concerning requirement for other functions such as significant storage, reproduction and optimal dark adaptation.

Previous data from this laboratory have shown that the minimum levels as defined above are not adequate for reproduction. Cows were able to produce live young at term but the calves were weak and soon died. Three to four times the minimum vitamin-A level beginning with the last month of pregnancy resulted in normal calves and the mothers supplied sufficient vitamin in the milk for normal growth of the calves for at least 3 months. Three to four times the minimum carotene level for the mother also resulted in birth of normal

calves. Deficiency symptoms, however, developed in the calves within 3 to 4 weeks although the cows remained normal.

Over extended periods of time levels of intake of about three times minimum vitamin-A intake resulted in storage. Five times the minimum carotene were required before significant storage could be demonstrated in rats after a 90-day dosage period. The evidence, therefore, indicates that about three times the minimum vitamin-A level and five times the minimum carotene level is about minimum for significant storage and reproduction and that with the aid of reserves established during non-lactating intervals should provide enough vitamin A in the milk to support young during the nursing period.

It is evident that the ratio of the relative efficiencies of vitamin A and of carotene widens with increasing levels of intake. On the basis of our analysis of U.S.P. Reference oil and its assigned biological value, 0.21 microgram approximates 1 I.U. At the biological unit level therefore the ratio of efficiency of vitamin A to carotene by weight is about 3:1; at the level to meet our definition of minimum about 6:1 and at the minimum level that results in significant storage and successful reproduction about 10:1. So far as mammals are concerned it is obvious that an international unit of carotene and an international unit of vitamin A have equality only under the conditions of the biological test. Double standards for requirements must be recognized, one for carotene and one for vitamin A, and both must be considered in evaluating the status of a dietary furnishing both sources.

The following human data that were reported in terms of intake per unit of body weight or suitable for calculating on this basis are briefly summarized for further comparison of general relationships. Aykroyd and Krishman ('36) reported eye symptoms in 27% of a group of 436 children who were receiving an estimated intake of 20–30 micrograms carotene per kilogram body weight. Edmund and Clemmesen ('37) found poor dark adaptation and some dysadaptation in men receiving about 18–20 I.U. per kilogram body weight. Jeghers ('37) likewise reported subjective night blindness in subjects

who for a long time had ingested daily an estimated 15–18 I.U. per kilogram body weight. Although these levels in some cases cannot be closely evaluated because both vitamin A and carotene contributed to the total, they appear to coincide with levels found by us to be borderline or deficient. Levels of intake three to five times our minimum and which suffice for

TABLE 4

*Summary of vitamin A and carotene requirements in relation to body weight and an illustration of requirements of a 70 kg. individual*

| REQUIREMENT | VITAMIN A PER KILOGRAM BODY WEIGHT | | CAROTENE PER KILOGRAM BODY WEIGHT | | TOTAL FOR 70 KG. INDIVIDUAL | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Vitamin A | | Carotene | |
| | *γ* | *I.U.* | *γ* | *I.U.* | *γ* | *I.U.* | *γ* | *I.U.* |
| Minimum for normal growth, freedom from clinical symptoms, little or no storage | 4 | 20 | 25 | 40 | 280 | 1400 | 1750 | 2800 |
| Minimum for significant storage, optimal dark adaptation, and reproduction | 12 | 60 | 125 | 200 | 840 | 4200 | 8750 | 14,000 |

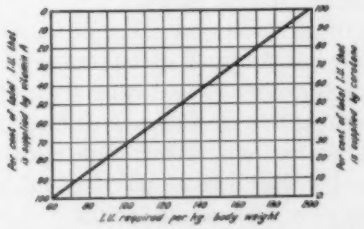

Fig. 1 Number of international units daily per kilogram body weight to satisfy minimum requirements for significant storage, optimal dark adaptation and reproduction when supplied by varying proportions of vitamin A and of carotene.

storage and reproduction have also been reported to be adequate for general well-being or for optimal dark adaptation in human subjects (Jeans, Blanchard and Zentmire, '37; Jeghers, '37; Lewis and Barenberg, '38; Booher, Callison and Hewston, '39). Crimm and Short ('37) and Phillips and Bohstedt ('38) presented data on dogs and rabbits respectively that fall within the range of intakes under discussion.

Because of the agreement of all these data, we propose and present in table 3 general daily requirements for vitamin A and for carotene and from these illustrate in table 4 that for a 70 kg. individual. The basic vitamin-A figures are given in round numbers converted to the basis of U.S.P. Reference oil. Figure 1 is presented to show the total requirement in international units to meet the minimum for significant storage, optimal dark adaptation and reproduction when supplied by varying proportions of vitamin A and of carotene.

## SUMMARY

Data are presented on time required to deplete vitamin-A reserves of horses and on their minimum requirement for vitamin A and for carotene. The more conspicuous symptoms of vitamin-A deficiency in the horse are described.

The data previously published on vitamin-A requirement of cattle, sheep, and swine were re-evaluated and a summary table presents all of the data from this laboratory on vitamin-A and carotene requirements of various species expressed both by weight and in international units.

The agreement of these and other data, with regard to the minimum to prevent earliest symptoms and also the higher levels necessary for storage and reproduction in animals and optimal dark adaptation in man are discussed. The necessity for two standards for expressing requirements, one for vitamin A and one for carotene is pointed out. These are illustrated by means of a table and a graph.

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